

NEUROSPORA CRASSA LACCASE: STRUCTURAL ORGANIZATION OF THE GENE AND REGULATION OF BIOSYNTHESIS

INAUGURAL-DISSERTATION

zur Erlangung der philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der
Universität Zürich

von
URSULA ANNA GERMANN
von Küsnacht/ZH und Gaiserwald/SG

Begutachtet von den Herren
PD Dr. K. Lerch
Prof. Dr. J.H.R. Kägi

Zürich 1987
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To Kerstin, Karl, and my parents

"Ja, wir werden alles, alles noch einmal in Frage stellen. Und wir werden nicht mit Siebenmeilenstiefeln vorwärtsgehen, sondern im Schneckentempo. Und was wir heute finden, werden wir morgen von der Tafel streichen und erst wieder anschreiben, wenn wir es noch einmal gefunden haben. Und was wir zu finden wünschen, das werden wir, gefunden, mit besonderem Misstrauen ansehen."..."Sollte uns dann aber jede andere Annahme als diese unter den Händen zerronnen sein, dann keine Gnade mehr mit denen, die nicht geforscht haben und doch reden."

Bertold Brecht "Galileo Galilei"

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SUMMARY

Copper is recognized as an essential trace metal and represents a prosthetic group in a number of proteins. Laccase (Benzenediol:oxygen oxidoreductase, EC 1. 10. 3. 2) is a multicopper oxidase and contains four copper ions per molecule. These can be assigned to three distinct copper centers which have previously been classified as type 1 or blue, type 2 or normal and type 3 or coupled binuclear. Laccase catalyzes the biological oxidation of organic substrates (e.g. mono-, di-, and polyphenols, aminophenols, diamines) with the concomitant complete reduction of molecular oxygen to water. The enzyme is widely distributed in plants and fungi, however, its biological function is still unclear. Although laccases have been purified from many different sources, no complete primary structure has yet been elucidated.

The ascomycete *Neurospora crassa* secretes laccase into the culture medium after induction with low amounts of protein synthesis inhibitors. The enzyme is a glycoprotein with a molecular weight of 64'000 and a carbohydrate content of 11-12% (Froehner, S. C. & Eriksson, K.-E. (1974) *J. Bacteriol.* 120, 458; Lerch, K., Deinum, J., & Reinhammar, B. (1978) *Biochim. Biophys. Acta* 534, 7). In this thesis the structural characterization of the laccase gene isolated from two different *N. crassa* wild type strains is reported. Moreover the regulation of laccase biosynthesis in vegetatively growing *N. crassa* cultures was investigated.

The laccase gene was cloned by cDNA synthesis with a laccase-specific undecadeoxynucleotide as primer and poly(A)RNA isolated from cycloheximide-treated *N. crassa* cultures as template. Based on the nucleotide sequence of the cDNA obtained a unique 21-mer was synthesized and used to screen a genomic DNA library from *N. crassa*. Five different positive clones were isolated and shown to share an overlapping region with the same pattern of restriction sites. Nucleotide sequence analysis revealed that they all contain part of the laccase gene, but lack the 5'end. A nick-translated laccase-specific DNA fragment was then used as a hybridization probe to generate and screen partial genomic DNA libraries



of *N. crassa*. In this way the full-length laccase gene was isolated from two unrelated *N. crassa* wild type strains. The presence of two different allelic forms was detected by sequence analysis. They conform to the same structural organization, but show an overall divergence of 5.3% which is mainly the result of point mutations in the non-translated regions. The coding region is interrupted by a short intron. The encoded protein chains differ in 12 out of 619 amino acid residues. The isoenzymes, however, do not differ functionally and display the same physicochemical properties.

Chemical analysis of the protein purified from the culture medium after induction with cycloheximide indicated a posttranslational processing of *N. crassa* laccase. The precursor exceeds the mature enzyme by 49 amino acids at its amino-terminal and 13 amino acids at its carboxyl-terminal end. The possible involvement of amino-terminal processing in secretion and of carboxyl-terminal processing in activation of the enzyme is discussed.

An amino acid sequence homology search revealed that the carboxyl-terminal region of *N. crassa* laccase exhibits a striking sequence homology to the carboxyl-terminal part of the third homology unit of the multicopper oxidase human ceruloplasmin, and to a lesser extent to the low molecular weight blue copper proteins plastocyanin and azurin. Based on an amino acid sequence comparison between these proteins putative copper ligands of *N. crassa* laccase are proposed.

The induction of laccase biosynthesis was studied in cycloheximide-treated vegetative cultures of *N. crassa*. A dramatic increase of the amounts of laccase mRNA was detected very shortly after administration of the inducing agent, suggesting that the *de novo* biosynthesis of *N. crassa* laccase is primarily regulated at the transcriptional level.

ZUSAMMENFASSUNG

Kupfer ist als ein wichtiges Spurenelement bekannt und stellt die prosthetische Gruppe verschiedener Proteine dar. Laccase (p-Diphenol:Sauerstoff Oxidoreduktase, EC 1. 10. 3. 2) gehört zur Klasse der Kupfer-enthaltenden Oxidoreduktasen und bindet vier Kupferionen pro Molekül. Diese werden drei verschiedenen Typen von Kupferzentren zugeordnet. Man unterscheidet Typ 1 oder das blaue Kupferzentrum, Typ 2 oder das normale Kupferzentrum und Typ 3 oder ein gekoppeltes Paar von Kupferionen. Laccase katalysiert die biologische Oxidation von organischen Substraten (z.B. Mono-, Di- und Polyphenole, Aminophenole, Diamine), wobei gleichzeitig molekularer Sauerstoff vollständig zu Wasser reduziert wird. Das Enzym wird vor allem von Pflanzen und Pilzen gebildet, seine biologische Funktion ist jedoch noch nicht geklärt. Obwohl das Protein aus verschiedenen Quellen rein dargestellt wurde, ist bis heute keine vollständige Primärstruktur bestimmt worden.

Nach Induktion durch Zugabe kleiner Mengen von Proteinsyntheseinhibitoren scheidet der Schimmelpilz *Neurospora crassa* Laccase ins Kulturmedium aus. Das Enzym ist ein Glykoprotein. Es besitzt ein Molekulargewicht von 64'000 und enthält zu 11-12% Kohlenhydrate (Froehner, S. C. & Eriksson, K.-E. (1974) *J. Bacteriol.* 120, 458; Lerch, K., Deinum, J. & Reinhammar, B. (1978) *Biochim. Biophys. Acta* 534, 7). In dieser Dissertation werden die Klonierung des Laccasegens aus zwei verschiedenen *N. crassa* Wildtyp-Stämmen und seine strukturelle Charakterisierung vorgestellt. Ausserdem wurde die Regulation der Biosynthese von Laccase in vegetativ gezüchteten *N. crassa* Kulturen untersucht.

Um das Strukturgen zu klonieren wurde ein spezifisches Undecadesoxy-nukleotid aus der teilweise bekannten Aminosäuresequenz von *N. crassa* Laccase abgeleitet und zur cDNA-Synthese eingesetzt. Als Matrize wurde Poly(A)RNA verwendet, die aus Cycloheximid-induzierten *N. crassa* Kulturen isoliert worden war. Nach Bestimmung der Nukleotidsequenz der cDNA wurde ein Laccase-spezifisches 21-mer synthetisiert. Dieses diente

als Hybridisierungsprobe zum Nachweis des Laccasegens bei der Untersuchung einer Genbank von *N. crassa*. Insgesamt wurden fünf verschiedene positive Klone isoliert. Durch Kartierung mit Restriktionsendonukleasen konnte ein allen gemeinsames Fragment gefunden werden, das einer Sequenzanalyse unterworfen wurde. Es stellte sich heraus, dass alle positiven Klone einen Teil des für Laccase kodierenden Strukturgens enthielten, jedoch in keinem das 5'-Ende vorhanden war. Sie lieferten aber ein spezifisches DNA-Fragment, das bei der Analyse gesamtzellulärer DNA zur Identifizierung des Laccasegens verhalf. Nach Herstellung von partiellen Genbanken, wurde das für Laccase kodierende Strukturgens aus zwei unabhängigen *N. crassa* Wildtyp-Stämmen isoliert. Eine Sequenzanalyse deutete auf das Vorhandensein von zwei unterschiedlichen Allelen. Beide Varianten des Gens unterstehen derselben strukturellen Organisation. Sie weisen jedoch eine gesamthafte Divergenz von 5.3% auf, die vor allem auf eine hohe Mutationsfrequenz in den untranslatierten Regionen zurückzuführen ist. Die kodierenden Regionen sind durch ein kleines Intron unterbrochen. In den aus 619 Aminosäuren aufgebauten Proteinketten sind total 12 Reste ausgetauscht. Die Isoenzyme besitzen jedoch dieselben katalytischen und ähnliche physikalisch-chemische Eigenschaften.

Laccase wurde aus dem Züchtungsmedium von induzierten *N. crassa* Kulturen gereinigt und mit proteinchemischen Methoden untersucht. Die erhaltenen Resultate zeigen, dass *N. crassa* Laccase als Vorläuferprotein mit erhöhtem Molekulargewicht gebildet wird. Der Vorläufer besitzt gegenüber dem reifen Enzym eine aminoterminal, sowie eine carboxylterminale Verlängerung von 49 resp. 13 Aminosäureresten. Vermutlich stellt das aminoterminal Präpeptid eine Signalsequenz dar, die zur Initiation der Sekretion notwendig ist, während eine Abspaltung der carboxylterminalen Verlängerung bei der Aktivierung des Enzyms von Bedeutung sein könnte.

Homologe Aminosäuresequenzen zur Primärstruktur von *N. crassa* Laccase wurden vor allem in Coeruloplasmin, einer Kupfer-enhaltenden Oxidase des Menschen, gefunden. Die carboxylterminale Region von Laccase zeigt eine auffallende strukturelle Aehnlichkeit zum carboxylterminalen Teil der

dritten Homologieuntereinheit von Coeruloplasmin. In einem kleineren Ausmass werden ausserdem homologe Segmente in den niedermolekularen blauen Kupferproteinen Plastocyanin und Azurin gefunden. Aufgrund eines Vergleichs der Aminosäuresequenzen der genannten Proteine wurden mögliche Kupferbindungsstellen in *N. crassa* Laccase postuliert. Die Resultate stützen zudem die Hypothese, dass die kleinmolekularen blauen Kupferprotein und die Kupfer-enthaltenden Oxidasen von einem gemeinsamen Urgen abstammen.

Die Induktion der Biosynthese von Laccase wurde in vegetativ gezüchteten *N. crassa* Kulturen studiert. Die Zugabe von kleinen Mengen Cycloheximid zu Kulturen in der logarithmischen Wachstumsphase bewirkt einen ausserordentlich raschen Anstieg der gebildeten Menge Laccase-mRNA. Die Resultate zeigen, dass die Biosynthese von *N. crassa* Laccase *de novo* erfolgt und primär einer transkriptionellen Kontrolle untersteht.

1. INTRODUCTION

1.1. Biochemical associations of copper

Copper plays an important role as a prosthetic group in a number of proteins. Only a few procaryotes, but no eucaryotes are known to survive without copper containing enzymes (Shapiro *et al.*, 1961; Porter *et al.*, 1962). As a transition element copper has the predominant ability to coordinate to biological ligands and to change its oxidation state easily, even when strongly chelated. Hence, this metal ion has particularly favorable properties to participate in electron transfer reactions (e.g. cytochrome oxidase, azurin, plastocyanin, stellacyanin) and to serve in oxygen transport (e.g. hemocyanin). Another primary role of copper is the protection of the cell against toxic intermediates which arise during partial reduction of molecular oxygen (e.g. Cu,Zn-superoxide dismutase). Many copper enzymes are known to be involved in the oxidation or hydroxylation of organic substrates (e.g. amine oxidases, ascorbate oxidase, ceruloplasmin, laccase, tyrosinase). Specific metal transport and storage proteins (e.g. ceruloplasmin, metallothionein) play an important homeostatic role in various organisms. Furthermore, copper proteins may also be involved in metal detoxification (e.g. metallothionein).

1.2. Multicopper oxidases are "blue" oxidases

Multicopper oxidases are oxidoreductases containing four or more copper ions per molecule (Fee, 1975). They catalyze the biological oxidation of organic substrates with the concomitant complete reduction of dioxygen to water. When dissolved in aqueous buffers multicopper oxidases have an unusually intense blue color that is associated with their enzymic activity. Therefore these enzymes are also called "blue" oxidases. The presence of a copper center that has previously been classified as type 1 (Malkin & Malmström, 1970) is responsible for the blue color.

All blue copper proteins are characterized by very unusual absorption features close to 600 nm ($\epsilon_{600} > 5 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, Malkin & Malmström, 1970; Gray & Solomon, 1981) and a unique EPR spectrum with a narrow hyperfine splitting in the $g \parallel$ region ($A_z < 10 \text{ mT}$; Malkin & Malmström, 1970; Malmström *et al.*, 1975; Reinhammar, 1979). The proteins with only one copper ion per molecule are known as small blue copper proteins (e.g. azurin, plastocyanin, stellacyanin; for reviews see Rydén, 1984; Adman, 1985) and function as electron carriers in different electron transport systems. Blue copper oxidases, however, bind at least four copper ions per molecule which can be assigned to three spectroscopically different copper centers. These are known as type 1 or blue, type 2 or non-blue, and type 3 or coupled binuclear (Malkin & Malmström, 1970). Type 2 copper is characterized by normal EPR parameters and by the ability to bind certain anions (e.g. F^-) that inhibit the oxidoreductase activity. Type 3 copper ions are EPR nondetectable, but have a strong absorption band in the near UV-spectral region that disappears upon reduction of the enzymes.

As summarized in Table 1, the multicopper oxidases ceruloplasmin, ascorbate oxidase, and laccase differ in metal stoichiometries and distribution of the distinct forms of copper present. Extensive studies of the spectroscopic, magnetic, and oxidation-reduction properties, however, showed striking similarities of these different macromolecules (for a review see Reinhammar & Malmström, 1981; Malmström *et al.*, 1975). Hence, even if ceruloplasmin, ascorbate oxidase, and laccase are different proteins a structural relatedness is indicated, which most probably corresponds to analogies in the catalytic mechanism.

Table 1: Copper content and distribution of three forms of copper in multicopper oxidases

Enzyme	Number of copper ions per molecule			References
	Type 1	Type 2	Type 3	
Ceruloplasmin	2	1	4	1; 2
Ascorbate oxidase	3	1	4	3
Tree laccase	1	1	2	4
Fungal laccase	1	1	2	5

- References: 1: Fee, 1975
2: Deinum & Vänngård, 1973
3: Marchesini & Kroneck, 1979
4: Malmström et al., 1968
5: Malmström et al., 1970

1. 3. Laccase

Laccase (benzenediol:oxygen oxidoreductase, EC 1. 10. 3. 2) binds four copper ions per molecule, representing one type 1, one type 2, and one type 3 copper center (Table 1). It provides the most simple system to study the active site structure and reactivity of multicopper oxidases.

1. 3. 1. Reactions catalyzed and inhibitors

During catalysis laccase utilizes the full oxidizing capacity of molecular oxygen which is reduced to two molecules of water. The enzyme shows a rather low degree of specificity with regard to the reducing substrate. A number of structurally different compounds including mono-, di-, and polyphenols, aminophenols, and diamines (Reinhammar, 1985) are readily oxidized. Monophenols need to be reacted in the presence of gelatine or detergents (e.g. Tween^R80), which protect the enzyme from inactivation by the substrate (Fahraeus, 1961). The *p*-diphenols (e.g. hydroquinone) are the most rapidly oxidized diphenols, but *o*-diphenols (e.g. catechol) and *m*-diphenols (e.g. resorcinol) are also oxidized at substantial rates (Fahraeus & Ljunggren, 1961). Some inorganic reducing agents such as hexacyanoferrate(II) are oxidized at comparable rates (Malmström *et al.*, 1969). Moreover, ascorbate represents a common substrate of all multicopper oxidases, although the specific activity of ascorbate oxidase (Stark & Dawson, 1963) is 10^3 - 10^4 x higher than those of laccase (Nakamura, 1960) and ceruloplasmin (Curzon & Young, 1972). The initial step of enzyme catalysis involves a one-electron transfer from the reducing substrate to Cu(II) type 1 of laccase (Reinhammar, 1985), resulting in the formation of a radical as the primary product.

The activity of laccase is strongly inhibited by a number of inorganic anions such as azide, cyanide, and fluoride (Reinhammar, 1985). Similar efficient inhibitors are thioglycolic acid and diethyldithiocarbamic acid, whereas EDTA affects laccase activity to a lesser extent (Bollag & Leonowicz, 1984).

1. 3. 2. Brief historical survey

Laccase was one of the first oxidative enzymes prepared from plant tissues. In 1883 Yoshida reported a thermolabile substance ("diastase") in the latex of the Japanese lacquer tree to be involved in the biological oxidation of urushiol and laccol, two catechol derivatives. A few years later, Bertrand (1894) identified the catalyst as an enzyme and named it laccase. He also introduced for the first time the concept of a metalloenzyme because he always found manganese copurifying with the protein. About 50 years later Kellin and Mann (1939) and Tissière (1948) established, however, that purified laccase does not bind manganese, but requires copper for its activity. Recent analysis of preserved samples of Bertrand's enzyme preparations by energy-disperse X-ray fluorescence indicated insufficient purity as the only reason for Bertrand's findings (Lehn *et al.*, 1986).

1. 3. 3. Biological distribution and function

Laccase is widely distributed in plants and microorganisms. No animal sources of the protein are known. Laccases have been isolated from higher plants including various species of the Japanese and Chinese lacquer trees (e.g. *Rhus vernicifera* and *Rhus succedanea*; Nakamura, 1958; Omura, 1961; Reinhammar, 1970). Furthermore, all members of the *Anacardiaceae*, including *Schinus molle* (Joel *et al.*, 1978), and suspension-cultured sycamore cells (*Acer pseudoplatanus*, Bligny & Douce, 1983) produce extracellular laccase. Laccase-like enzymes have also been prepared from peaches (*Prunus persica*; Mayer & Harel, 1968; Lehman *et al.*, 1974) and tea leaves (*Camellia sinensis*; Gregory & Bendall, 1966). The main sources for laccases, however, are many different fungi. In ascomycetes the enzyme is found either in constitutive intracellular forms (e.g. *Podospora anserina*, Esser, 1963) or as an inducible exoenzyme (e.g. *Neurospora crassa*, Froehner & Eriksson, 1974a,b; Lerch *et al.*, 1978). In *Aspergillus nidulans* laccase levels are developmentally regulated (Law & Timberlake, 1980). In many Basidiomycete species the enzyme occurs both in inducible

and constitutive forms (Leonowicz & Trojanowski, 1975), intracellularly (Leonowicz *et al.*, 1978) and extracellularly (Bollag & Leonowicz, 1984). Laccases have been identified in *Polyporus versicolor* (Mosbach, 1963), *Agaricus bisporus* (Wood, 1980), *Schizophyllum commune* (Leonard, 1971), *Fomes annosus* (Haars *et al.*, 1981), *Pholiota mutabilis* (Leonowicz & Grzywnowicz, 1981), *Pleurotus ostreatus* (Leonowicz *et al.*, 1978), and the soil fungus *Rhizoctonia praticola* (Bollag *et al.*, 1979). Laccase was furthermore shown to be present in the wild mushroom *Russula foetens* (Gregg & Miller, 1940) and the deuteromycete wine fungus *Botrytis cinerea* (Dubernet *et al.*, 1977; Gigi *et al.*, 1981).

Our knowledge about the biological functions of laccase is quite incomplete mainly because the enzyme has multiple functions depending on the source. The role of laccase in the *Rhus* species is probably best understood. When a lacquer tree becomes damaged the white latex containing laccase and various phenols (e.g. urushiol and laccol) seeps out. In contact with the air the enzyme is activated and catalyzes the oxidation of the phenolic compounds to free radicals, which subsequently polymerize to form a protective natural plastic (Reinhammar, 1985).

Because of its abundant occurrence in wood-destroying Basidiomycetes (white-rot fungi), which are able to decompose lignin, laccase has been suggested to play a major role in lignin degradation (for a review see Kirk, 1971). This idea is supported by studies with a laccase-less mutant of the white-rot fungus *Sporotrichum pulverulentum* (Ander & Eriksson, 1976) and by the absence of laccase in brown-rot fungi, which are unable to decompose lignin and desintegrate cellulose only (Rypáček, 1966; Kirk, 1973). Fungal laccase has been shown to catalyze the demethylation of lignin and lignin-related model compounds, a reaction which is suggested to be an initial step in lignin biodegradation (Trojanowski *et al.*, 1966; Ishihara & Miyazaki, 1974). Moreover, fungal laccase was also proposed to be responsible for side-chain elimination reactions which could occur during lignin degradation (Kirk *et al.*, 1968; Ishihara & Miyazaki, 1974). Incubations of isolated lignin (milled wool lignin) with fungal laccase mainly led to polymerization reactions and only minor formation of low molecular weight products was observed (Ishihara & Miyazaki, 1972). Purified laccase

Table 2: Molecular weights and carbohydrate contents of laccases

Source	Molecular weight	% Carbohydrates	References
<u>Rhus vernicifera</u>	110'000 - 140'000	45	Nakamura, 1958; Reinhammar 1970
<u>Rhus succedanea</u>	130'000	n. d.	Omura, 1961
<u>Prunus persica</u>	70'000 - 90'000	n. d.	Mayer & Harel, 1968
<u>Acer pseudoplatanus</u>	97'000	45	Bligny & Douce, 1983
<u>Schinus molle</u>	96'000 - 105'000	47 - 62	Bar-Nun et al., 1981
<u>Podospora anserina I</u>	390'000	24	Molitoris, 1976
<u>Podospora anserina II</u>	70'000	25	Molitoris, 1976
<u>Podospora anserina III</u>	80'000	23	Molitoris, 1976
<u>Neurospora crassa</u>	64'800	11 - 12	Frøehner & Eriksson 1974b
<u>Aspergillus nidulans</u>	85'000	n. d.	Law & Timberlake, 1980
<u>Polyporus versicolor A</u>	64'400	10 - 14	Fahraeus & Reinhammar, 1967
<u>Polyporus versicolor B</u>	64'700	10	Mosbach, 1963
<u>Agaricus bisporus</u>	100'000	n. d.	Wood, 1980

was also reported to introduce carboxyl-groups into milled wool lignin and thus to increase its solubility in water (Konishi *et al.*, 1974). It is apparent that laccase activity is needed during wood decay, however, the exact role of the enzyme in lignin degradation is still unknown.

Fungal laccases have also been proposed to be involved in the formation of humic acids in the soil (Lindqvist & Lindqvist, 1969). In *Aspergillus nidulans* laccase appears to be responsible for a step in the synthesis of conidial pigments (Clutterbuck, 1972).

1. 3. 4. Molecular properties

Laccases have been isolated from many different sources. It is generally accepted that the enzymes bind four copper ions per molecule and are glycoproteins. Data on molecular weight and carbohydrate content of some well-defined laccases are summarized in Table 2. A great variety in molecular weight ranging from 64'000 to 140'000 daltons is indicated. However, it is presumed that the main difference is due to variations in carbohydrate content. Hence, all these enzymes, including the subunit of *Podospora* laccase I, probably consist of a single polypeptide chain with a length of about 500 amino acid residues (see Table 3). The quantitative distribution of the different sugars has been determined for several laccases including *Rhus* (Mayer & Harel, 1979), *Schinus molle* (Bar-Nun *et al.*, 1981), *Polyporus* (Fahraeus & Reinhammar, 1967), *Neurospora* (Frøehner & Eriksson, 1974b), and *Podospora* (Minuth *et al.*, 1978) laccase. However, no structure of any carbohydrate moiety was characterized completely. Also the possible functions of the carbohydrate part of laccase remains to be defined.

Similarly the protein part of the molecule has not been analyzed in great details. Several groups reported amino acid compositions of laccases. Some of these are summarized in Table 3. The three forms (I, II, and III) of *Podospora* laccase (Molitoris, 1976) and the two forms (A and B) of *Polyporus* laccase (Fahraeus & Reinhammar, 1967; Briving, 1975) were demonstrated to differ in amino acid analysis and are therefore indicated to be encoded by separate genes.

Table 3: Amino acid composition of laccases

	<u>Rhus</u> ¹	<u>Podospora</u> ²			<u>Polyporus</u> ³	<u>Neurospora</u> ^{4:5}	
		I	II	III	A		
Asx	60	57	60	60	79	77	78
Thr	50	28	36	48	41	34	34
Ser	37	22	25	32	35	27	28
Glx	37	45	25	48	27	35	29
Pro	38	35	34	30	42	33	32
Gly	38	35	34	30	42	33	32
Ala	39	27	30	29	57	35	32
Val	43	22	34	28	35	32	31
Cys	7	19	11	18	6	2	6
Met	11	18	9	7	4	6	8
Ile	31	32	20	24	30	27	30
Leu	34	28	29	39	35	38	39
Tyr	24	13	14	15	15	20	22
Phe	30	18	16	19	31	19	17
Lys	26	17	13	8	6	20	20
His	17	18	16	17	17	16	14
Arg	13	22	17	17	14	21	21
Trp	6	24	13	19	4	26	16
Total	539	484	439	503	521	516	503

- References: 1: Reinhammar, 1970
 2: Minuth et al., 1978
 3: Fahraeus & Reinhammar, 1967
 4: Froehner & Eriksson, 1974b
 5: Lerch et al., 1978

Whereas the complete amino acid sequence of the multicopper oxidase human ceruloplasmin has been determined (Takahashi *et al.*, 1984), surprisingly only very limited data are available describing the primary structure of laccases. In a preliminary sequence analysis of laccase B from the white-rot fungus *Polyporus versicolor* (Briving *et al.*, 1980) three cysteine-containing fragments have been characterized.

1. 4. *Neurospora crassa* laccase

The ascomycete *Neurospora crassa* represents a genetically well-defined organism which has been often used in biochemical and molecular biological studies. The fungus produces several copper binding proteins including Cu,Zn-superoxide dismutase (Misra & Fridovich, 1972; Rapp *et al.*, 1972), cytochrome oxidase (Bertrand & Werner, 1979), copper metallothionein (Lerch, 1980), tyrosinase (Fling *et al.*, 1963), and laccase (Froehner & Eriksson, 1974b). The primary structures of Cu,Zn-superoxide dismutase (Lerch & Schenk, 1985), copper metallothionein (Lerch, 1980), and tyrosinase (Lerch, 1982; Lerch *et al.*, 1982) have been elucidated. Moreover the molecular cloning of the genes coding for several cytochrome oxidase subunits (Burger *et al.*, 1982; Browning & RajBhandary, 1982; Sachs *et al.*, 1986) and copper metallothionein (Münger *et al.*, 1985) have been reported.

N. crassa laccase activity was discovered by Horowitz & Fling (1953) when they investigated the genetic determination of tyrosinase thermostability. The enzyme, described as a second phenoloxidase, was mainly found in the culture medium and shown to oxidize dihydroxyphenylalanine. However, it differed from tyrosinase in its inability to use tyrosine as a substrate. Also the thermostability of the two enzymes was not affected in the same way (Stadler, 1954). By examination of its substrate specificity and its spectrum of inhibitors, the newly found phenoloxidase was classified as laccase (Sueoka, 1959). More than a decade later the induction of *N. crassa* laccase was studied (Froehner & Eriksson, 1974a) and some properties of the purified enzyme were reported (Froehner & Eriksson,

1974b; Lerch *et al.*, 1978). The structural gene coding for laccase seems to be repressed during asexual growth of *N. crassa*. Vegetative liquid cultures do not produce significant amounts of laccase except when treated with small amounts of RNA or protein synthesis inhibitors (cycloheximide > actinomycin D > puromycin) or when induced with aromatic compounds (2,5-xyldine > o-toluidine $\approx$ p-cresol). In contrast to the white-rot fungus *Polyporus versicolor* (Grabbe *et al.*, 1968) no induction of *N. crassa* laccase is achieved in response to agents which uncouple oxidative phosphorylation (e.g. 2,4-dinitrophenol) or lignin and phenolic lignin degradation products. Due to similarities of conditions used for induction in vegetative *N. crassa* cultures the *de novo* synthesis of both laccase and tyrosinase were suggested to be controlled by a common mechanism. To explain the apparent paradox of the induction of enzyme biosynthesis with protein synthesis inhibitors Horowitz and coworkers (Horowitz *et al.*, 1970b) proposed the following model. In rapidly growing *N. crassa* cultures the transcription of the structural gene is repressed by a very unstable, hence continuously synthesized repressor protein. Addition of small amounts of agents which interact with protein synthesis lowers the concentration of active repressor molecule. As a result the structural gene is derepressed and transcribed freely. High concentrations of protein synthesis inhibitors, however, block enzyme production, too. The hypothetic metabolically unstable repressor protein which is supposed to control tyrosinase and/or laccase gene expression has not been isolated, to date.

Under physiological conditions tyrosinase synthesis is induced either after starvation of vegetative *N. crassa* cultures (Horowitz *et al.*, 1974a) or in cultures undergoing sexual differentiation (Hirsch, 1954). A preliminary study (Cruz & Terenzi, 1981) suggests that the mechanism of tyrosinase derepression under conditions of starvation differs from the one in response to mating. Induction of *N. crassa* laccase has so far not been studied under physiological conditions. Also the biological function of this enzyme is unknown.

After treatment of vegetative *N. crassa* cultures with inducers most of *de novo* synthesized laccase (65-90%) is secreted into the culture medium. The substrate specificity of extracellular *N. crassa* laccase is comparable to other laccases and the enzyme typically binds four copper ions per molecule (see section 1.3.). *N. crassa* laccase is a monomeric glycoprotein with an apparent M_r of 64 kD and a carbohydrate content of 11-12%. Preliminary analysis of the carbohydrate composition revealed a high content of mannose and small amounts of two sugars which were not identified. Two different groups (Froehner & Eriksson, 1974b; Lerch *et al.*, 1978) reported very similar amino acid compositions of *N. crassa* laccase (see Table 3). Only the values for glutamic acid, cysteine, methionine, and tryptophan differ by more than 10%.

1. 5. Purpose of this study

In order to determine the primary structure of *N. crassa* laccase and to study its molecular mechanisms of biosynthesis and secretion the main purpose of this thesis was to clone the structural gene. The laccase gene was isolated from two different *N. crassa* wild type strains and its structural organization elucidated. Extracellular *N. crassa* laccase was investigated by protein chemical analysis and the data obtained were compared with the amino acid sequence deduced from the nucleotide sequence of the gene. Moreover, the induction of laccase biosynthesis was studied in vegetative *N. crassa* cultures.

2. RESULTS

2. 1. Induction of *N. crassa* laccase

The induction of extracellular laccase by addition of cycloheximide to rapidly growing 2-day old cultures of *N. crassa* wild type strain TS is presented in Fig. 1. In control cultures no laccase activity was detected in the culture medium at any growth phase of the organism. In induced cultures growth was inhibited by approximately 70% during the first 48 h after addition of cycloheximide. Furthermore cells started autolysis at the end of the time period observed. The addition of cycloheximide led to a rapid increase of production of extracellular laccase after a short lag period (~ 10 h). 96 h after induction laccase activity reached a maximum level and the culture medium was collected for preparation of the enzyme.

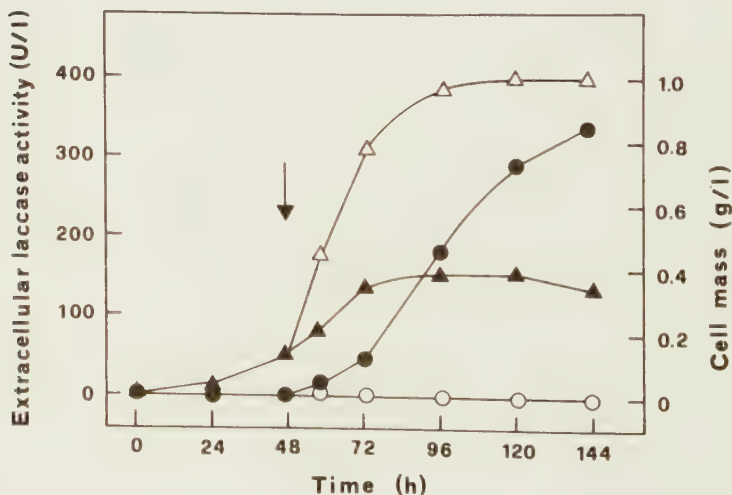


Fig. 1: induction of extracellular laccase in *N. crassa* wild type strain TS. Cycloheximide was added 48 h after inoculation (arrow) at a concentration of 2.8 μ M. Growth (dry weight) of control (Δ) and induced ($\blacktriangle$) cultures and laccase activity in the corresponding culture medium (control, $\circ$; induced, $\bullet$) were followed as a function of time.

2. 2. Isolation of poly(A)RNA

Total cellular RNA was isolated from *N. crassa* wild type strain TS grown for 96 h. Laccase synthesis was induced 48 h after inoculation by addition of cycloheximide at a concentration of 2.8 μ M (see Fig. 1). No influence of this protein synthesis inhibitor on the amounts of total cellular RNA was detected. Usually 20 mg RNA were extracted from 1 g mycelia of both induced and non-induced state. By selective precipitation 75% of high molecular weight RNA were recovered. Poly(A)RNA was purified by oligo(dT)cellulose chromatography. Nearly 4% of the RNA applied were obtained by the first passage and 50% were recovered when the first eluate was reapplied. In order to estimate the content of laccase mRNA poly(A)RNA was used to direct protein synthesis in a rabbit reticulocyte lysate system. *In vitro* synthesized laccase was analyzed by double immunoprecipitation with anti-laccase antibodies and protein A from *Staphylococcus aureus*, followed by SDS polyacrylamide gel electrophoresis (Fig. 2). No translatable laccase mRNA was detected in non-induced control cultures. However, considerable amounts of laccase mRNA were shown to be present in induced cultures. A single translation product with an apparent M_r of 61 kD was recognized by anti-laccase antibodies.

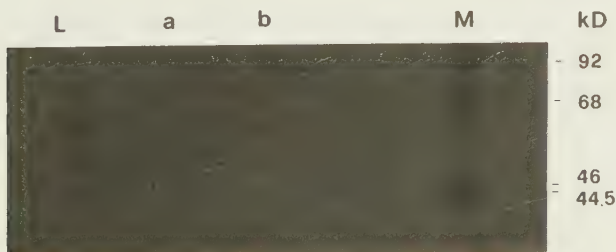


Fig. 2: Immunological analysis of *in vitro* translation products with anti-laccase antibodies. Poly(A)RNA used to direct protein synthesis was isolated from cycloheximide-induced (lane a) and non-induced (lane b) *N. crassa* cultures. 'L' indicates a marker of purified 14 C-carboxymethylated apolaccase. 'M' denotes molecular weight marker proteins. Experimental details are given under materials and methods.

2. 3. Synthesis of laccase specific cDNA by primer extension

In order to design a unique oligodeoxynucleotide suitable for priming laccase-specific cDNA synthesis, carboxymethylated apoprotein was digested with trypsin. Some of the peptides were isolated by HPLC and subjected to sequence analysis (E. Schenk & K. Lerch, unpublished results). From the tryptic decapeptide T_5 with the amino acid sequence Ala-Phe-Asn-Asp-Asn-Cys-Asp-Ala-Trp-Arg (Fig. 3A) a unique 11-mer (Fig. 3C) coding for residues 5-8 within the peptide was deduced. This primer was selected over the other seven possibilities (Fig. 3B) based on the published codon usage in other nuclear genes of *N. crassa* coding for *trp-1* (Schechtman & Yanofsky, 1983), histones H3 and H4 (Woudt *et al.*, 1983), and glutamate dehydrogenase (Kinnaird & Fincham, 1983). Furthermore, possible mismatches between the 11-mer and laccase mRNA were only of G·U type, building a 'wobble' base pair (Crick, 1966) which has been shown to approximate an A·U base pair in stabilizing efficiency (Romaniuk *et al.*, 1979). Poly(A)RNA prepared from cycloheximide-induced *N. crassa* cultures served as a template for 11-mer-primed cDNA synthesis. Analysis of the primer extension products by denaturing polyacrylamide gel electrophoresis (Fig. 4A) showed a pattern of more than ten discrete single-stranded cDNA bands with an estimated maximal length of > 1 kb. Only high molecular weight primer extension products obtained by gel filtration on Sephacryl S-300 (Fig. 4B) were subjected to chemical nucleotide sequencing (Fig. 3D). The amino acid sequence deduced from the specifically primed cDNA (Fig. 3E) was in complete agreement with the partially known primary structure of *N. crassa* laccase from the priming site towards the amino-terminus (Fig. 3A). This allowed the synthesis of a unique 21-mer (Fig. 3F) which was used as a hybridization probe to screen a genomic DNA library of *N. crassa*.

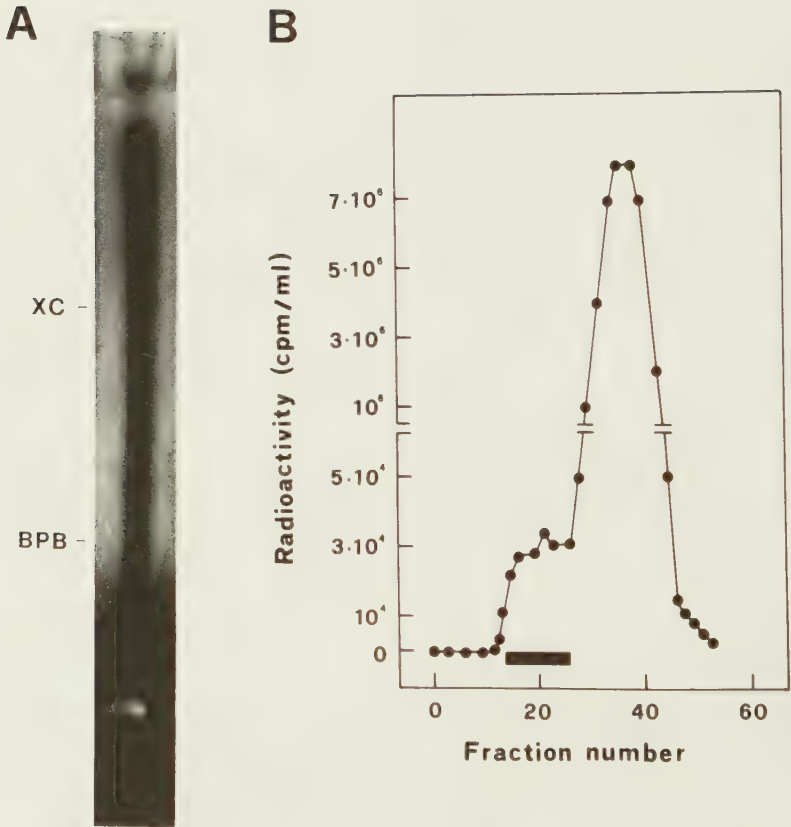


Fig. 4: Analysis of 11-mer primed cDNA synthesis. Poly(A)RNA isolated from cycloheximide-treated *N. crassa* cultures was used as a template.

(A): Single stranded cDNA was size-fractionated on a 42% (w/v) urea/6% (w/v) polyacrylamide gel. Bromophenol blue (BPB) and xylene cyanol (XC) were used as markers (Maniatis *et al.*, 1982).

(B): High molecular weight primer extension products (indicated by the bar) were isolated by gel filtration on Sephacryl S-300 and subjected to sequence analysis by the chemical cleavage method. Experimental details are given under materials and methods.

2. 4. Screening of a genomic *N. crassa* DNA library and characterization of positive clones

A partial *Sau*3A genomic DNA library in the plasmid vector pRK-9 (Schechtman & Yanofsky, 1983) was kindly provided by M. G. Schechtman (Syracuse University, NY, USA) and used to transform *E. coli* LE392 cells. By screening of 60'000 colonies with the laccase specific 21-mer (Fig. 3F) more than 100 were found giving an enhanced hybridization signal at low stringency washing conditions (6 x SSC, 42°C; Fig 5A). However, rescreening of these colonies with the same hybridization probe at higher stringency (6 x SSC, 45-60°C, Fig. 5B) yielded only seven positive clones (#10, #53, #62, #81, #101, #102, and #113). Examination of their plasmid DNA by restriction endonuclease digestions revealed two pairs of them to be identical (#53 and #81, #102 and #113). Restriction maps of the five positive clones #10, #53, #61, #101, and #102 are presented in Fig. 6. Although all positive clones differ in size and part of the pattern of restriction sites, they share an overlapping DNA region of 2.0 kb, including a 1.3 kb *Sall* fragment.

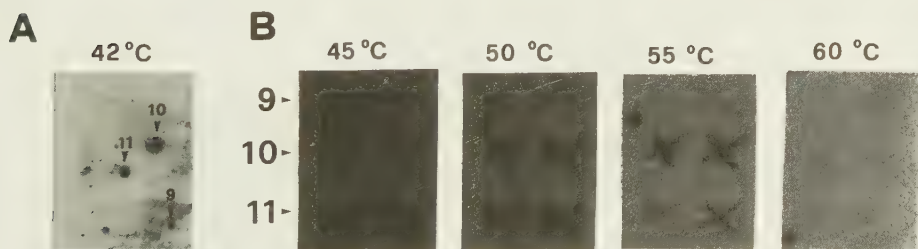


Fig. 5: Screening and rescreening of clones #9, #10, and #11.

- (A): The genomic *N. crassa* DNA library was screened with ^{32}P -labeled 21-mer at low stringency washing conditions (6 x SSC, 42°C) and possible positive clones (e.g. #9, #10, #11) were picked from the masterplate.
- (B): Clones #9, #10, and #11 were replated and two randomly chosen single colonies were streaked on nitrocellulose. The filters were rescreened with ^{32}P -labeled 21-mer and washed in 6 x SSC by raising the temperature (45-60°C). Experimental details are given in materials and methods.

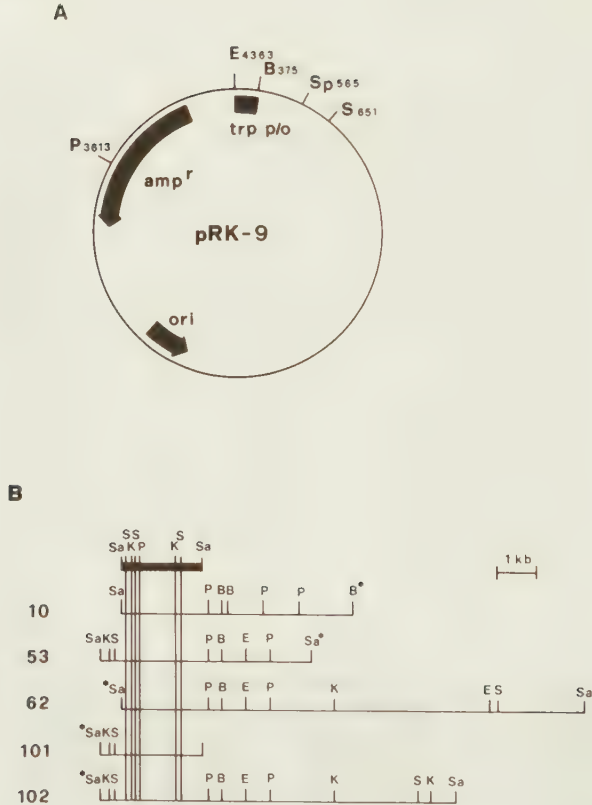


Fig. 6: Restriction maps of the five positive clones #10, #53, #62, #101, and #102.

- (A): Restriction map of the parental plasmid pRK-9, a derivative of pBR322 (Bolivar *et al.*, 1977). The 375 bp EcoRI-BamHI fragment was substituted with the promoter/operator region of the *trp* gene from *Serratia marcescens* (Miozzari & Yanofsky, 1978). To generate a DNA library *N. crassa* Sau3A fragments were inserted into the BamHI site of pRK-9 (Schechtman & Yanofsky, 1983). Ori is the origin of replication, *amp^r* the gene coding for ampicillin resistance. Numbers refer to sequence positions in pBR322.
- (B): Restriction maps of *N. crassa* DNA inserts in the five positive clones. The common 2.0 kb DNA region is presented on top and indicated by a bar. The Sau3A sites close to the EcoRI site of the cloning vector pRK-9 are marked with asterisks. Restriction endonucleases are abbreviated as follows: B: BamHI, E: EcoRI, K: KpnI, P: PstI, S: Sali, Sa: Sau3A, Sp: SphI.

SalI restriction endonuclease digests of the five positive clones are shown in Fig. 7. Only the common 1.36 kb SalI fragment strongly hybridized to the ^{32}P -labeled 21-mer. Hence, this fragment was structurally further characterized with the strategy outlined in Fig. 8. Both the chemical cleavage and the chain-termination technique were used for DNA sequence analysis and every sequence was determined at least twice. The 1.36 kb SalI fragment was found to contain an open reading frame of 726 nucleotides starting with the triplet GTC of one SalI restriction site and ending with the termination signal TAG (Fig. 9). The correct 21-mer (Fig. 3F) used to screen the *N. crassa* DNA library could be localized readily. Moreover, the amino acid sequence deduced from the coding region agreed completely with the primary structures of six previously isolated tryptic peptides ($\text{T}_1\text{-T}_6$, E. Schenk & K. Lerch, unpublished results) of *N. crassa* laccase. Hence, the results clearly indicated that part of the laccase gene had been characterized. Further sequence analysis in direction towards the 5'end of the structural gene, however, revealed that none of the five positive clones contained the whole coding region. Most interesting, all *N. crassa* DNA inserts ended at only two particular Sau3A sites as suggested by the restriction maps presented in Fig. 6.

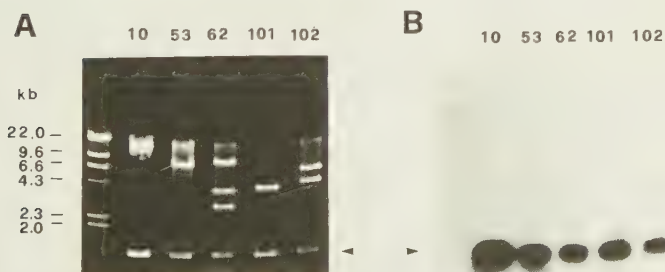


Fig. 7: Digestion of plasmid DNA isolated from the five positive clones #10, #53, #62, #101, and #102 with SalI and hybridization to labeled 21-mer.

(A): Fragments were size-fractionated by gel electrophoresis on 1% (w/v) agarose and stained with ethidium bromide.

(B): DNA was transferred to nitrocellulose (Southern *et al.*, 1975) and hybridized to ^{32}P -labeled 21-mer. The lengths of size markers (HindIII-digested phage λ DNA) are given in kb.

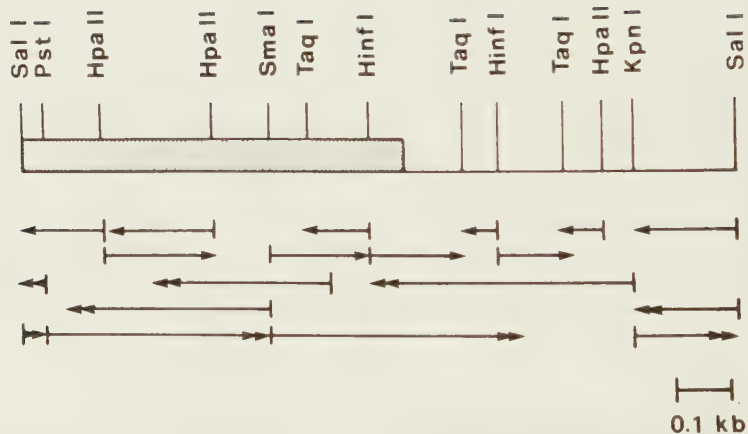


Fig. 8: Strategy of sequence analysis of the 1.36 kb SalI fragment common to the five positive clones #10, #53, #62, #101, and #102. Only non-frequent restriction sites and the sites used for sequence analysis are shown. Arrows indicate direction and extent of sequencing. Both the chemical cleavage (→) and the chain-termination technique (|→) were used. The bar indicates the coding region.

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Fig. 9: Primary structure of the coding part found within the 1.36 kb SalI fragment (Fig. 8) and deduced amino acid sequence. The nucleotides complementary to the 21-mer (Fig. 3F), which was used to screen the genomic N. crassa DNA library, are boxed. Bars indicate tryptic peptides (T₁ - T₆) that were isolated previously from carboxymethylated apolaccase and characterized by amino acid and sequence analysis.

2. 5. Molecular cloning of the laccase gene of two different *N. crassa* wild type strains

In order to clone the full-length gene coding for laccase genomic DNA was isolated from the two *N. crassa* wild type strains OR (74-OR8-1a, FGSC #988, Fungal Genetics Stock Center, Kansas City, KS, USA) and TS (69-1113, provided by N. H. Horowitz, Pasadena, CA, USA). The genomic DNA of both strains was analyzed by digestions with various restriction endonucleases, followed by Southern transfer and hybridization to a laccase specific DNA fragment. As shown in Fig. 10 each restriction enzyme yielded only a single fragment giving rise to a hybridization signal.

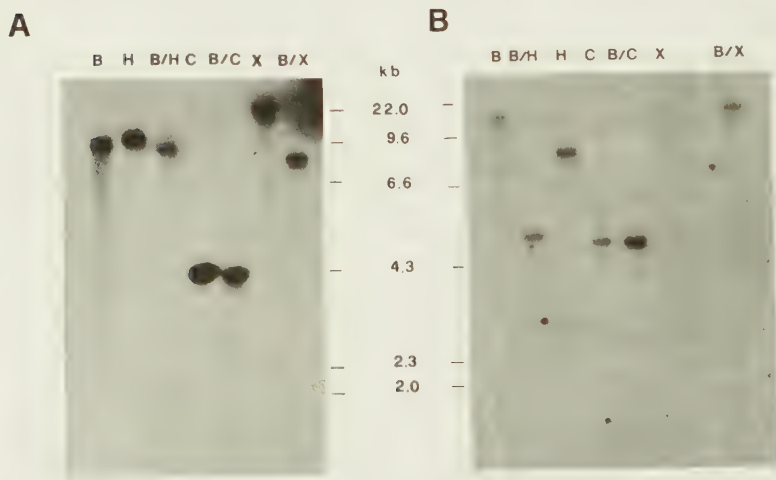


Fig. 10: Southern hybridization (Southern, 1975) of different restriction endonuclease digests of genomic DNA isolated from the *N. crassa* wild type strains OR (A) and TS (B). The blots were hybridized with the nick-translated 1.36 kb *SalI* fragment (Fig. 8) and washed in 0.1 x SSC, 0.5% (w/v) SDS at 68°C. Restriction endonucleases used are abbreviated as follows: B: *Bam*HI, C: *Cla*I, H: *Hind*III, X: *Xho*I.

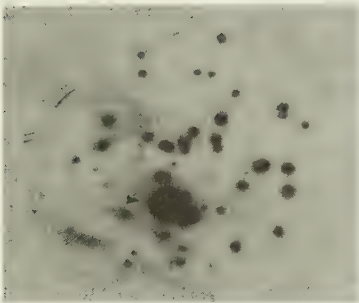
Partial genomic DNA libraries of the two wild type strains OR and TS were then prepared as follows.

Genomic DNA of *N. crassa* strain OR was digested with BamHI and fragments with a length of 8-10 kb were inserted into pBR322. After transformation of *E. coli* LE392 approximately 200 colonies were screened with the nick-translated 1.36 kb SalI fragment (see Fig. 8) and the positive clone pORL-1 was isolated (Fig. 11A).

Similarly the laccase gene of *N. crassa* strain TS was cloned. Genomic DNA was double-digested with BamHI and HindIII and fragments ranging from 4-6 kb were cloned into pUC8 by transforming *E. coli* JM83. Screening of about 400 colonies with the ^{32}P -labeled 1.36 kb SalI fragment resulted in the isolation of the positive clone pTSL-1 (Fig. 11B).

The two positive clones pORL-1 and pTSL-1 were characterized by restriction mapping as presented in Figs. 12 and 13, respectively.

A



B



Fig. 11: Isolations of clones pORL-1 (A) and pTSL-1 (B). Partial genomic *N. crassa* DNA libraries (see text for details) were screened with the nick-translated 1.36 kb SalI fragment (Fig. 8). Filters were washed in 1 x SSC, 0.5% SDS (w/v) at 65°C. For experimental details see materials & methods.

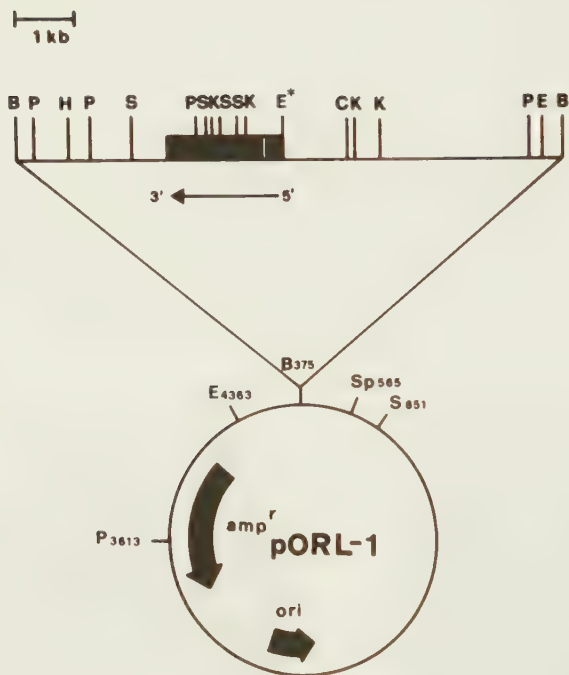


Fig. 12: Restriction map of pORL-1. The parental plasmid is pBR322 (Bolivar et al., 1977). The *N. crassa* DNA was inserted into the BamHI site of the vector as described in the text. The bars indicate the two exons of the laccase gene. Ori is the origin of replication, amp^r the gene coding for ampicillin resistance. Numbers refer to the sequence positions in pBR322. Restriction enzymes are abbreviated as follows: B: BamHI; C: ClaI; E: EcoRI; E*: Eco RI*; H: HindIII; K: KpnI; P: PstI; S: SalI.

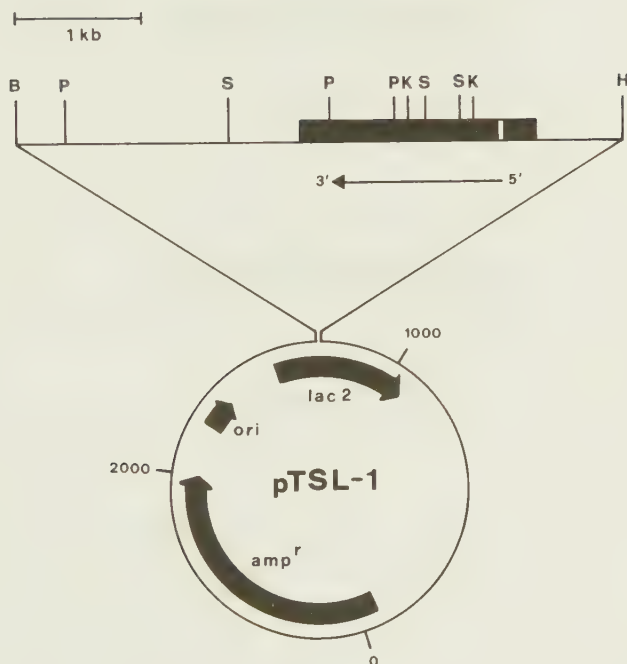


Fig. 13: Restriction map of pTSL-1. The parental plasmid is pUC8 (Vieira & Messing, 1982). The *N. crassa* BamHI-HindIII fragment was inserted into the vector as described in the text. The bars indicate the two exons of the laccase gene. Ori is the origin of replication, lac 2 the β -galactosidase gene, amp^r the gene coding for ampicillin resistance. Numbers refer to sequence positions in pUC8. Restriction sites are abbreviated as follows: B: BamHI; H: HindIII; K: KpnI; P: PstI; S: SalI.

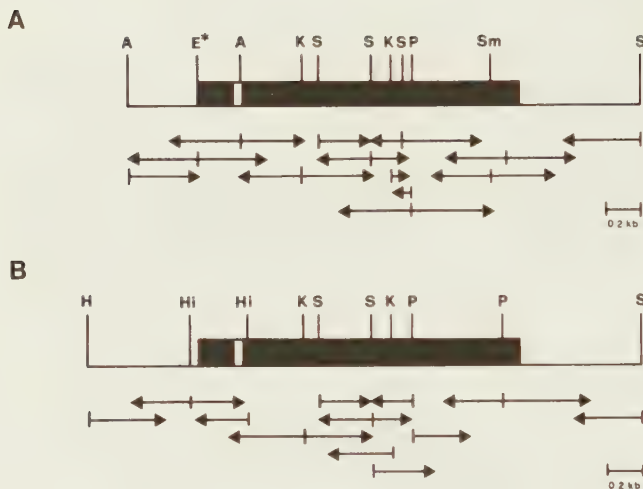


Fig. 14: Strategy of sequence analysis of the laccase gene isolated from *N. crassa* wild type strain OR (A) and TS (B). Bars indicate exons, arrows the direction and extent of chain-termination sequencing (Sanger et al., 1979). Only restriction sites used for cloning of isolated subfragments in the single-stranded vectors M13mp18 or M13mp19 (Norrander et al., 1983) are shown. They are abbreviated as follows: A: AluI; E*: EcoRI*; H: HindIII; Hi: HindII; K: KpnI; P: PstI; S: SalI; Sm: SmaI.

(next three pages)

Fig. 15: Comparison of the primary structures of the laccase genes isolated from *N. crassa* wild type strains OR and TS. Only substitutions and deletions (-) are shown. Numbers refer to the nucleotide sequence and deduced amino acid sequence found in wild type strain TS. Numberings start at the major transcription initiation site and at the amino-terminus of extracellular laccase, respectively. A promoter element, the TATAAA sequence (Breathnach & Chambon, 1981) is boxed. Major and minor transcription initiation sites are indicated by ▼, consensus sequences within the intron (see Table 11) are underlined and the polyadenylation site is marked with an arrow. Potential glycosylation sites are marked by asterisks.

```

-200 -150
OR
TS CAGCTCAGCCTCACCATACGAAGGACCTGGTACTCCACGAGGACGAGGTGGTAAGCGCCATCCACTTTGCGATTGATGACCCAAAGACCTCGG

-100 -50
OR
TS CTGCTCCACAACACCTCAACATGCAAGGGCAAGGTAAGTGGTACTCATCTGCTTGTCAACGTTATATAAGTAGTTCAGATCTCTCCATTCTGGTGG

+1 50 75 -----
OR
TS TCTTTCGATTGCTGTTCTCATCATCATATCAGGTCACTCTCCAGACTCGGAGGTGAAGTCCAACCATCACATACCTTTCTTTTGGATTATATCTTC

200 150
OR
TS -G TCCT----- T C A C TT C C G A GA T T
TCTCCCAACCCCTCTCTCCCATCTTCAATCCCTCATATCAGTCAATCCAAGCCCTGTTGACTCTCTTCATCACTTCCACTCGCGABAATCTTCATC

193 250
OR
TS ATCACC ATG AAA TTC TTG GGC ATT GCC GCC CTC GTT GCG GGC CTT CTT GCC CCT TCA CTT GTT CTC GGT GCT CCT
Met Lys Phe Leu Gly Ile Ala Ala Leu Val Ala Gly Leu Leu Ala Pro Ser Leu Val Leu Gly Ala Pro
-49 -40 -30

300
OR
TS GCA CCT GGC ACC GAG GGA GTG AAT CTC CTC ACT CCC GTT GAT AAG AGA CAG GAC TCC CAA GCC GAA CGT TAT GGA
Ala Pro Gly Thr Glu Gly Val Asn Leu Leu Thr Pro Val Asp Lys Arg Gln Asp Ser Gln Ala Glu Arg Tyr Gly
-20 -10

350 400
OR
TS GGT GGC GGA GGC GGT GGT TGC AAC TCC CCG ACC AAC CGG CAG TGT TGG TCT CCA GGA TTC AAC ATC AAC ACC GAC
Gly Gly Gly Gly Gly Cys Asn Ser Pro Thr Asn Arg Gln Cys Trp Ser Pro Gly Phe Asn Ile Asn Thr Asp
+1 10 20

450
OR
TS TAT GAA CTC GGA ACT CCT AAT ACC GGC AAG ACC AGA CGG GTAAGTAAGGCTCCATATTACCGGTGCAGAGAAGCAATGACTGA
Tyr Glu Leu Gly Thr Pro Asn Thr Gly Lys Thr Arg Arg
30 37

500 550
OR
TS CAGCTCATCTAG TAC AAA CTC ACC CTC ACC GAG ACT GAT AAC TGG CTT GGC CCT GAT GGT GTC ATA AAG GAT AAG GTC
Tyr Lys Leu Thr Leu Thr Glu Thr Asp Asn Trp Leu Gly Pro Asp Gly Val Ile Lys Asp Lys Val
38 50

600
OR
TS ATG ATG GTC AAC GAC AAC ATC ATT GGA CCT ACC ATT CAG GCT GAT TGG GGC GAC TAT ATT GAG ATC ACC GTC ATC
Met Met Val Asn Asn Asn Ile Ile Gly Pro Thr Ile Gln Ala Asp Trp Gly Asp Tyr Ile Glu Ile Thr Val Ile
60 70 80

650 700
OR
TS AAC AAA CTG AAG TCG AAC GGC ACC TCG ATC CAC TGG CAT GGC ATG CAC CAG CGC AAC TCC AAC ATC CAG GAC GGC
Asn Lys Leu Lys Ser Asn Gly Thr Ser Ile His Trp His Gly Met His Gln Arg Asn Ser Asn Ile Gln Asp Gly
90 100

750
OR
TS GTC AAC GGC GTT ACC GAG TGC CCC ATC CCA CCC AGA GGC GGC AGC AAA GAT CAC TGC TGG CGG GCC ACC CAG TAC
Val Asn Gly Val Thr Glu Cys Pro Ile Pro Pro Arg Gly Gly Ser Lys Val Tyr Arg Trp Arg Ala Thr Gln Tyr
110 120 130

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800 850
OR C
TS GGG ACG TCG TGG TAC CAC TCG CAT TTC TCA GCG CAG TAC GGC AAT GGC ATT GTT GGG CCC ATT GTT ATC AAC GGA
OR
TS Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly Ile Val Gly Pro Ile Val Ile Asn Gly
140 150

900 C T
OR
TS CCG GCC TCG GCC AAC TAC GAC GTC GAC CTC GGC CCT TTC CCG CTC ACG GAC TAC TAT TAC GAC ACA GCC GAC CGT
OR
TS Pro Ala Ser Ala Asn Tyr Asp Val Asp Leu Gly Pro Phe Pro Leu Thr Asp Tyr Tyr Tyr Asp Thr Ala Asp Arg
160 170 180

950 1000 T
OR
TS CTC GTG CTG CTC ACA CAG CAT GCC GGC CCT CCG CCG AGC AAC AAT GTG CTC TTC AAC GGC TTC GCC AAG CAT CCG
OR
TS Leu Val Leu Leu Thr Gln His Ala Gly Pro Pro Ser Asn Asn Val Leu Phe Asn Gly Phe Ala Lys His Pro
190 200

1050 C T
OR
TS ACC ACA GGC GCT GGC CAG TAT GCT ACC GTG TCG CTC ACC AAG GGC AAG AAG CAT CCG CTG CCG CTC ATC AAC ACC
OR
TS Thr Thr Gly Ala Gly Gln Tyr Ala Thr Val Ser Leu Thr Lys Gly Lys Lys His Arg Leu Arg Leu Ile Asn Thr
210 220 230

1100 1150
OR T T A
TS TCG GTC GAG AAC CAC TTC CAA CTG TCG CTG GTG AAT CAC TCC ATG ACC ATC TCG GCG GAT CTG GTT CCG GTG
OR
TS Ser Val Glu Asn His Phe Gln Leu Ser Leu Val Asn His Ser Met Thr Ile Ile Ser Ala Asp Leu Val Pro Val
240 250

1200 T C G T T
OR
TS CAG CCC TAC AAG GTC GAC AGC CTG CTG CTC GGC ATC GGC CAG CGC TAC GAC GTC ATC ATC GAC GCC AAC CAG GCC
OR
TS Gln Pro Tyr Lys Val Asp Ser Leu Leu Gly Ile Gly Gln Arg Tyr Asp Val Ile Ile Asp Ala Asn Gln Ala
260 270 280

1250 1300 A G G A G G GAT C C
OR
TS GTT GGG AAC TAC TGG TTC AAT GTG ACG TTT GGT GGC AAC GAC CTA TGC GGT ACC TCG GAC AAC AAG TAC CCG GCC
OR
TS Val Gly Asn Tyr Trp Phe Asn Val Thr Phe Gly Gly Asn Asp Leu Cys Gly Thr Ser Asp Asn Lys Tyr Pro Ala
290 300

1350 C G GTC
OR
TS GCC ATC TTC CGG TAC CAG GGC GCG CCA AAG GCA CTG CCG ACC AAC AAG GGC GTT GCG CCG CCT GAC CAC CAA TGC
OR
TS Ala Ile Phe Arg Tyr Gln Gly Ala Pro Lys Ala Leu Pro Thr Asn Lys Gly Val Ala Pro Pro Asp His Gln Cys
310 320 330

1400 1450
OR
TS CTG GAC CTC AAC GAC CTG AAG CCC GTC CTG CAG CGC AGC CTG AAC ACC AAC AGC ATC GCA CTC AAC ACT GGC AAC
OR
TS Leu Asp Leu Asn Asp Leu Lys Pro Val Leu Gln Arg Ser Leu Asn Thr Asn Ser Ile Ala Leu Asn Thr Gly Asn
340 350

1500
OR
TS ACG ATC CCC ATC ACG CTC GAC GGG TTC GTC TGG CGG GTC AAT GGA ACG GCC ATC AAC ATC AAC TGG AAC AAG CCG
OR
TS Thr Ile Pro Ile Thr Leu Asp Gly Phe Val Trp Arg Val Asn Gly Thr Ala Ile Asn Ile Ash Trp Asn Lys Pro
360 370 380

1550 1600 . . .
 OR C
 TS GTG CTG GAG TAC GTG ATG ACG GGC AAC ACC AAC TAC TCG CAG TCC GAC AAC ATT GTG CAG GTC GAG GGC GTC AAC
 OR Leu
 TS Val Leu Glu Tyr Val Met Thr Gly Asn Thr Asn Tyr Ser Gln Ser Asp Asn Ile Val Gln Val Glu Gly Val Asn
 390 * 400

1650
 OR C
 TS CAA TGG AAG TAC TGG CTC ATC GAG AAC GAC CCC GAT GGT GCC TTT AGC CTG CCG CAT CCC ATC CAC CTG CAC GGC
 OR
 TS Gln Trp Lys Tyr Trp Leu Ile Glu Asn Asp Pro Asp Gly Ala Phe Ser Leu Pro His Pro Ile His Leu His Gly
 410 420 430

1700 1750 . . .
 OR C
 TS CAC GAC TTT CTC ATC CTC GGT CGG TCG CCC GAC GTG ACC GCC ATC AGC CAG ACG CGC TAC GTG TTT GAC CCG GCC
 OR
 TS His Asp Phe Leu Ile Leu Gly Arg Ser Pro Asp Val Thr Ala Ile Ser Gln Thr Arg Tyr Val Phe Asp Pro Ala
 440 450

1800
 OR
 TS GTG GAC ATG GCT CGG TTG AAC GGC AAC AAC CCG ACG CGG CGC GAC ACG GCC ATG CTC CCG GCC AAG GGC TGG CTG
 OR
 TS Val Asp Met Ala Arg Leu Asn Gly Asn Asn Pro Thr Arg Arg Asp Thr Ala Met Leu Pro Ala Lys Gly Trp Leu
 460 * 470 480

1850 1900 . . . A
 OR G
 TS CTC ATC GCG TTC CGC ACC GAC AAT CCC GGC TCG TGG CTG ATG CAC TGC CAC ATT GCC TGG CAC GTG TCG GGG GGT
 OR
 TS Leu Ile Ala Phe Arg Thr Asp Asn Pro Gly Ser Trp Leu Met His Cys His Ile Ala Trp His Val Ser Gly Gly
 490 500

1950
 OR A G
 TS CTG TCG AAC CAG TTC CTG GAG CGG GCG CAG GAC CTC AGG AAC AGT ATC AGC CCT GCA GAT AAG AAG GCC TTT AAC
 OR
 TS Leu Ser Asn Gln Phe Leu Glu Arg Ala Gln Asp Leu Arg Asn Ser Ile Ser Pro Ala Asp Lys Ala Phe Asn
 510 520 530

2000 2050 . . . C
 OR
 TS GAC AAC TGT GAC GCG TGG AGG GCG TAC TTC CCT GAC AAT GCG CCG TTC CCC AAG GAC GAC TCT GGC CTC AGG AGT
 OR
 TS Asp Asn Cys Asp Ala Trp Arg Ala Tyr Phe Pro Asp Asn Ala Pro Phe Pro Lys Asp Asp Ser Gly Leu Arg Ser
 540 550

2100 2150 . . . T T
 OR T G G AT T G T
 TS GGC GTC AAG GCA AGA GAG GTG AAG ATG AAG TGG TAG AGCGGTGATTCCCAAGATGATATAATGATTGAATGGCCAAATAG
 OR
 TS Gly Val Lys Ala Arg Glu Val Lys Met Lys Trp ***
 560 570

2200 2250
 OR G G C T TT AAGCTCTT
 TS GGTGTATATATTGCGCTGGTGTGGTCTTGGTAATACGTAATCTCTCTCCCTTTTCTTACTTT-----CCCTTCATCGACCTTTGGACTTGC

2300
 OR C A TATTA
 TS AACACTTGTGTTTTTGGCTGATGGTCTAAGCAAAGATTTTGAGATTGAAGAGTCGGAGCTATAGAAGATGATATTA----GTTGAGCCGACGAGG

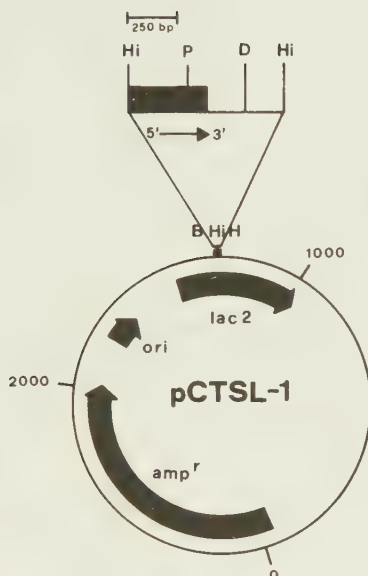
2350 2400 . . .
 OR CA CG A GT TCCT C T C TC TCCT C A G A
 TS GGTGAATGATATTGTACGTATACAATTGACGAGTGATCTCTCTCTCTGTTT-CTTAGCTGATGCTCCTTTCTCGCGGATAGTCAGGGTCCAT

2.6. Structure and organization of the laccase gene

The strategies employed for sequence analysis of the laccase genes isolated from the *N. crassa* wild type strains OR and TS are shown in Fig. 14. The complete primary structures of the two homologous genes were elucidated using the chain-termination technique (Sanger *et al.*, 1979). In general, every sequence was reconfirmed by a second analysis. The nucleotide sequences obtained were analyzed with a computer program (Queen & Korn, 1980). The structures of the genes coding for laccase of *N. crassa* wild type strains OR and TS together with 5' and 3' flanking regions are aligned in Fig. 15. The start of transcription was determined by nuclease S1 mapping. A singly ^{32}P -end labeled restriction fragment (marked at the *Sau*961 site (GGNCC) at position +225 and ending at the *Rsa*I site (GTAC) at position -72) was isolated from clone pTSL-1 and hybridized to poly(A)RNA extracted from *N. crassa* strain TS. After treatment with nuclease S1 three resistant fragments were observed ending at C +1, T +2, and A +75, hence, indicating a major and a minor transcription initiation site.

In order to determine the transcription termination site double stranded cDNA was prepared using poly(A)RNA isolated from induced *N. crassa* wild type strain TS as template and oligo(dT) as primer for first strand synthesis. Double stranded cDNA was tailed with dGTP and inserted into pUC8 that had previously been linearized with *Hind*II and tailed with dCTP. *E. coli* JM83 cells were transformed and screened with nick-translated 1.36 kb *Sal*I fragment (Fig. 8). Seven positive clones (pCTSL-1 to pCTSL-7) were isolated from about 600 colonies investigated, indicating that the poly(A)RNA used for cDNA synthesis contained approximately 1% laccase mRNA. The length of the *N. crassa* cDNA inserts in the seven positive clones was estimated by agarose gel electrophoresis and ranged from 200 bp to 700 bp. The largest cDNA clone pCTSL-1 was structurally further analyzed by restriction mapping and nucleotide sequencing as shown in Fig. 16. Clone pCTSL-1 contained the 3'terminal coding part of the laccase gene starting from position +1960 and ending at the polyadenylation site at position +2391.

A



B

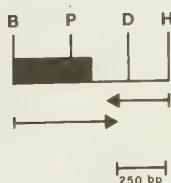


Fig. 16: Restriction map of *N. crassa* laccase cDNA clone pCTSL-1 (A) and strategy used for sequence analysis (B).

- (A): The parental plasmid is pUC8 (Vieira & Messing, 1982). The *N. crassa* cDNA was inserted into the single HindIII site as described in the text. The bar represents the coding region of the laccase gene. Ori is the origin of replication, lac 2 the β -galactosidase gene. Amp^r is the gene coding for ampicillin resistance. Numbers refer to sequence positions of pUC8. Restriction sites are abbreviated as follows: B: BamHI; D: DdeI; H: HindIII; Hi: HindII, P: PstI.
- (B): The chemical cleavage technique (Maxam & Gilbert, 1980) was used to analyze the nucleotide sequence. Arrows indicate direction and extent of sequencing.

A high degree of variability of the *N. crassa* laccase gene is indicated by the sequence comparison shown in Fig. 15. Numbers and types of point mutations are summarized in Table 4. Main differences are found in the non-translated regions. On the 5'flanking side between the major transcription startpoint (+1) and the codon for the initiator methionine (+193) 15 bases are deleted and 27 are exchanged when the gene of wildtype strain OR is compared to that of wild type strain TS. Within the 3'flanking region from the termination codon (+2109) to the polyadenylation site (+2391) 13 insertions, one deletion, and 26 substitutions can be detected. Moreover, five out of 57 nucleotides in the intron are exchanged. However, differences are also manifested in the coding part, indicating laccase to represent an example of polymorphism in *N. crassa*. Whereas 31 base changes occur at the third position of codons and do not cause changes of amino acids, 19 nucleotide substitutions lead to the replacement of 12 amino acids as listed in Table 5.

Table 4: Numbers and types of substitutions in the structural genes coding for laccase in N. crassa wild type strains OR and TS

Region	OR	T	C	G	A	TS
5'Flanking		0	3	2	1	T
		6	0	4	2	C
(1 - 192)		0	0	0	2	G
		3	2	2	0	A
Intron		0	1	0	1	T
		1	0	0	0	C
(451 - 507)		0	0	0	1	G
		1	0	0	0	A
3'Flanking		0	5	3	0	T
		5	0	1	1	C
(2110 - 2391)		1	0	0	3	G
		5	1	1	0	A
Coding		0	7	1	0	T
	12	0	6	2		C
	0	3	0	5		G
	0	4	10	0		A

Table 5: Point mutations in replacement sites of the laccase genes in N. crassa wild type strains OR and TS

Strain OR		Strain TS	
Codon	Amino acid	Amino acid	Codon

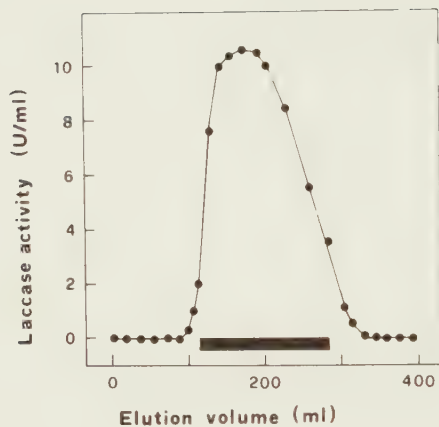
ATT	Ile 49	:	Leu 49	CTT
AAG	Lys 65	:	Asn 65	AAC
TTG	Leu 243	:	Ser 243	TCG
TTC	Phe 268	:	Leu 268	CTG
GTC	Val 271	:	Ile 271	ATC
AGC	Ser 297	:	Asn 297	AAC
AAG	Lys 298	:	Asp 298	GAC
GAT	Asp 302	:	Thr 302	ACC
CAC	His 306	:	Lys 306	AAG
CAG	Gln 325	:	Lys 325	AAG
GTC	Val 330	:	Pro 330	CCT
CTG	Leu 390	:	Met 390	ATG

2. 7. Primary structure of extracellular *N. crassa* laccase

Extracellular laccase was analyzed by protein chemical methods in order to determine the amino- and carboxyl-terminal amino acids and to confirm part of the primary structure deduced from the nucleotide sequence of the gene (see Fig. 15). Laccase was isolated from the culture medium harvested 96 h after induction of *N. crassa* wild type strain TS with cycloheximide (see Fig. 1). The protein was purified by gel filtration on Sephadex G-100, followed by chromatography on hydroxyapatite as demonstrated in Fig. 17. Copurifying brownish pigments were then separated by passage through DEAE-Sephadex and the blue copper protein was recovered again by chromatography on hydroxyapatite. Usually two peaks containing laccase activity were eluted from the first hydroxyapatite column. Their amino acid compositions were established to be very similar (K. Lerch, unpublished results), indicating that they may represent laccase molecules with different carbohydrate content. Further experiments were carried out using only laccase eluting with the first peak. The overall yield of purified laccase was 40% and the enzyme displayed a specific activity of 41 units/mg.

Ten fragments including free homoserine lactone (Table 6) were expected to be obtained by cyanogen bromide cleavage of laccase, as suggested by the amino acid sequence deduced from the nucleotide sequence of the structural gene (Fig. 15). The predicted carboxyl-terminal dipeptide Lys-Trp could never be isolated despite many attempts, indicating a carboxyl-terminal processing of the translation product. Hence, both the amino- and the carboxyl-terminal cyanogen bromide fragments were expected to contain two Cys-residues. In order to label these amino acid residues reduced apolaccase was modified by S-carboxymethylation using iodo(2-¹⁴C)acetic acid. The cleavage products obtained by subsequent cyanogen bromide treatment were size fractionated by gel filtration as shown in Fig. 18. The amino-terminal (CB-N) as well as the carboxyl-terminal (CB-C) cyanogen bromide fragment could be isolated by HPLC of pooled fractions #101 - #106. The elution profile of an analytical run is presented in Fig. 19. Both polypeptides were identified by amino acid analysis (Table 7).

A



B

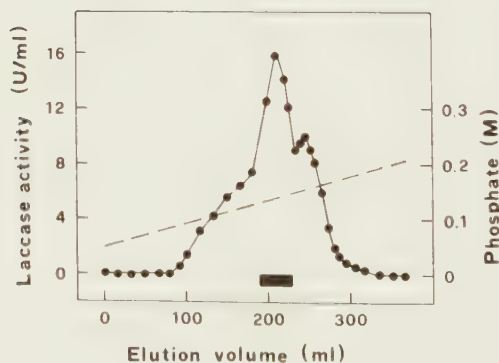


Fig. 17: isolation. of *N. crassa* laccase.

(A): Sephadex G-100 gel filtration of evaporated and dialyzed culture medium.

(B): Following hydroxyapatite chromatography. The column was eluted with a linear sodium phosphate gradient (dashed line). Bars indicate the pooled fractions.

Experimental details are given in materials and methods.

Table 6: Cyanogen bromide fragments predicted by the amino acid sequence deduced from the primary structure of the laccase gene of N. crassa wild type strain TS (Fig. 15)

No.	Fragment ends	Length	Molecular weight
I	-49 - 60	109	11'478
II	61	1	149
III	62 - 99	38	4'251
IV	100 - 249	150	16'522
V	250 - 390	141	15'446
VI	391 - 462	72	8'143
VII	463 - 477	15	1'687
VIII	478 - 498	21	2'387
IX	499 - 568	70	8'124
X	569 - 570	2	333

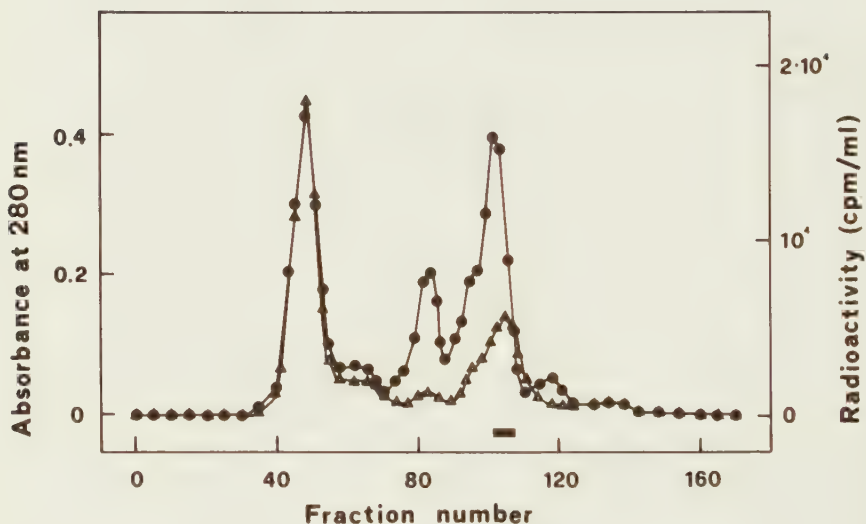


Fig. 18: Size fractionation of cleavage products obtained by cyanogen bromide treatment of (^{14}C)-carboxymethylated apolaccase. The Sephadex G-50 column (2.5 x 150 cm) was eluted with 7% (v/v) formic acid at a rate of 19.2 ml/h. The collected fractions had a volume of 4.8 ml. Their absorbance was recorded at 280 nm (●). Fragments containing Cys-residues were detected by measuring radioactivity (Δ). The bar marks the fractions which were pooled for the isolation of the amino- and carboxyl-terminal cyanogen bromide fragment by HPLC (see Fig. 19).

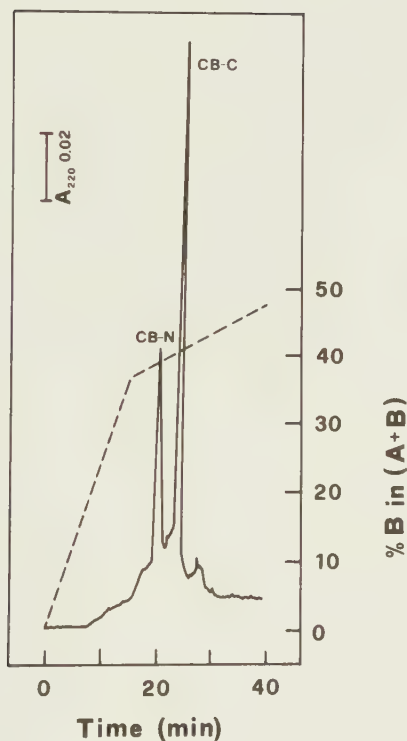


Fig. 19: Isolation of the amino-terminal (CB-N) and the carboxyl-terminal (CB-C) cyanogen bromide fragment of *N. crassa* laccase by HPLC. An analytical run is shown. 0.45 ml of the pool obtained by Sephadex G-50 gel filtration (see Fig. 18) were applied. Chromatography was performed on a Vydac C4 column (4.6 internal diam. x 250 mm) at a flow rate of 0.7 ml/min using a gradient (dashed line) formed between buffer A (0.1% (v/v) TFA) and buffer B (same as A, containing 60 % (v/v) acetonitrile).

Table 7: Amino acid compositions of amino-terminal (CB-N) and carboxyl-terminal (CB-C) cyanogen bromide fragments of extracellular laccase. They were isolated by HPLC as shown in Fig. 19. Numbers in paranthesis refer to values predicted by the primary structure deduced from the nucleotide sequence of the gene.

	CB-N		CB-C	
Asx	10.9	(10)	12.8	(12)
Thr	6.4	(8)	0.0	(0)
Ser	2.2	(2)	4.7	(5)
Glx	3.4	(3)	3.0	(3)
Pro	3.8	(4)	3.7	(4)
Gly	9.8	(10)	2.9	(3)
Ala	0.2	(0)	6.2	(7)
Val	1.7	(2)	1.0	(1)
Cys	n.d.	(2)	n.d.	(2)
Met	n.d.	(1)	n.d.	(0)
Ile	2.2	(2)	2.3	(2)
Leu	3.8	(4)	3.7	(4)
Tyr	n.d.	(2)	n.d.	(1)
Phe	1.1	(1)	3.8	(4)
Lys	4.0	(4)	3.0	(3)
His	n.d.	(3)	n.d.	(3)
Arg	2.6	(3)	2.8	(3)
Trp	n.d.	(2)	n.d.	(2)
Total		(60)		(59)

Peptide maps obtained by HPLC of chymotryptic digestion of the amino-terminal (CB-N) and tryptic digestion of the carboxyl-terminal (CB-C) cyanogen bromide fraction are presented in Figs. 20 and 21, respectively. The amino acid compositions of the purified peptides are summarized in Tables 8 and 9. All the chymotryptic and tryptic peptides could be easily aligned with the primary structure of laccase as deduced from the nucleotide sequence of the gene (Fig. 22). The data indicate both an amino-terminal and a carboxyl-terminal processing of the translation product. Native laccase isolated from the culture medium of cycloheximide-induced *N. crassa* cultures had earlier been shown to possess a blocked amino-terminus (K. Lerch, unpublished results). Therefore the suggested amino-terminal chymotryptic peptide NC-5 was subjected to amino-terminal analysis. In fact, no modified Gly-residue was detected after dansylation of the polypeptide, followed by hydrolysis and HPLC analysis. Moreover, the peptide was not susceptible to Edman degradation (Edman & Begg, 1967). The nature of the blocking group, however, has not been determined.

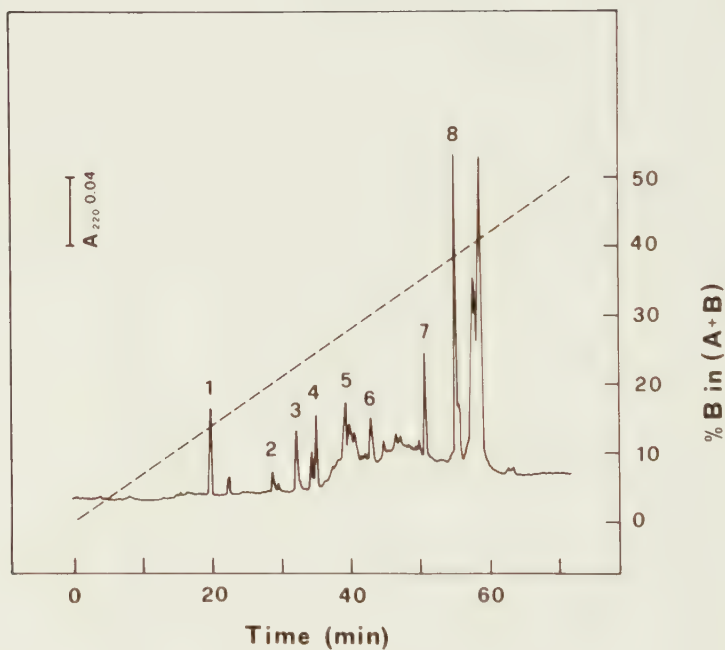


Fig. 20: Chymotryptic peptide map of the amino-terminal cyanogen bromide fragment (CB-N, see Fig. 19) of *N. crassa* laccase. Chromatography was carried out on an Aquapore RP-300 column (4.6 internal diam. x 250 mm) at a flow rate of 0.7 ml/min using a linear gradient formed by buffer A (0.1% (v/v) TFA) and buffer B (same as A, containing 60 % (v/v) acetonitrile). The amino acid analysis of the peptides (NC 1-8) is shown in Table 8.

Table 8: Amino acid compositions of chymotryptic peptides of the amino-terminal cyanogen bromide fragment(CB-N, Fig. 19). Peptides were isolated by HPLC (Fig. 20). Numbers in paranthesis refer to values predicted by the primary structure deduced from the nucleotide sequence of the gene.

	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6	NC-7	NC-8
Asx	-	-	2.9 (3)	1.0 (1)	1.6 (2)	2.2 (2)	2.8 (3)	2.2 (2)
Thr	1.9 (2)	-	1.1 (1)	2.9 (3)	1.1 (1)	-	1.1 (1)	2.9 (3)
Ser	-	1.0 (1)	-	-	0.9 (1)	-	1.0 (1)	-
Glx	-	-	-	1.3 (1)	1.3 (1)	-	-	1.1 (1)
Pro	-	1.0 (1)	-	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)	-
Gly	1.2 (1)	1.1 (1)	-	2.2 (2)	5.0 (5)	2.0 (2)	1.0 (1)	-
Val	-	-	-	-	-	1.6 (2)	-	-
Cys	-	-	-	-	n.d.(2)	-	-	-
Met	-	-	-	-	-	n.d.(1)	-	-
Ile	-	-	1.2 (1)	-	-	0.8 (1)	1.1 (1)	-
Leu	-	-	-	1.0 (1)	-	1.2 (1)	-	1.8 (2)
Tyr	0.6 (1)	-	0.7 (1)	0.7 (1)	-	-	0.7 (1)	-
Phe	-	0.9 (1)	-	-	-	-	1.0 (1)	-
Lys	1.2 (1)	-	-	1.0 (1)	-	2.2 (2)	-	1.0 (1)
Arg	1.9 (2)	-	-	2.0 (2)	1.1 (1)	-	-	-
Trp	-	-	-	-	n.d.(1)	-	-	n.d.(1)
Total	(7)	(4)	(6)	(13)	(15)	(12)	(10)	(10)

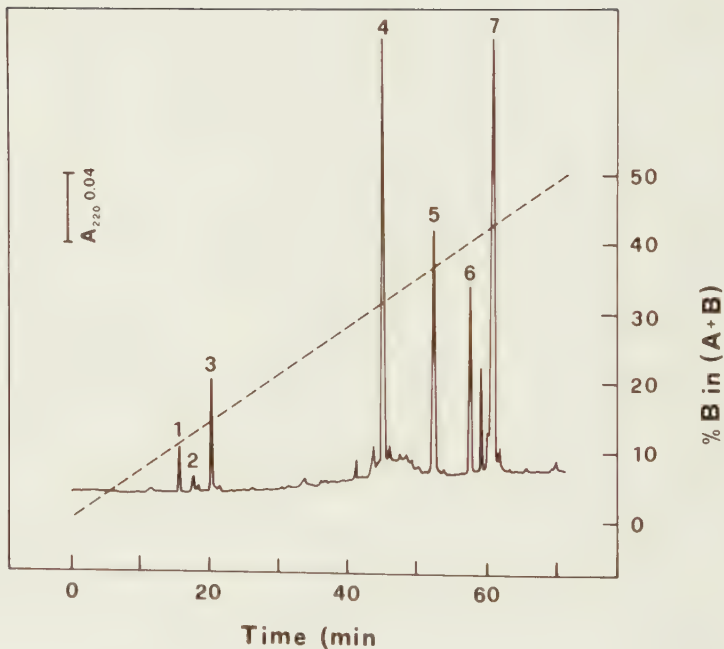


Fig. 21: Tryptic peptide map of the carboxyl-terminal cyanogen bromide fragment (CB-C, see Fig. 19) of *N. crassa* laccase. Chromatography was performed as described in Fig. 20. The peptides (CT 1-7) were characterized by amino acid analysis as summarized in Table 9.

Table 9: Amino acid compositions of tryptic peptides of the carboxyl-terminal cyanogen bromide fragment(CB-C, Fig. 19). Peptides were isolated by HPLC (Fig. 21). Numbers in paranthesis refer to values predicted by the primary structure deduced from the nucleotide sequence of the gene.

	CT-1	CT-2	CT-3	CT-4	CT-5	CT-6	CT-7
Asx	1.0 (1)	1.9 (2)	1.8 (2)	4.2 (4)	2.2 (2)	4.3 (4)	1.1 (1)
Thr	-	-	-	-	-	-	-
Ser	-	1.1 (1)	1.9 (2)	-	-	1.1 (1)	2.1 (2)
Glx	1.1 (1)	-	-	-	-	-	2.1 (2)
Pro	-	-	1.1 (1)	-	3.0 (3)	2.8 (3)	-
Gly	-	1.0 (1)	-	-	-	1.0 (1)	1.9 (2)
Ala	1.0 (1)	-	1.1 (1)	1.9 (2)	2.0 (2)	2.0 (2)	1.0 (1)
Val	-	-	-	-	-	-	0.9 (1)
Cys	-	-	-	n.d.(1)	-	-	n.d.(1)
Ile	-	-	1.1 (1)	-	-	-	1.2 (1)
Leu	0.9 (1)	1.0 (1)	-	-	-	1.0 (1)	1.9 (2)
Tyr	-	-	-	-	n.d.(1)	n.d.(1)	-
Phe	-	-	-	0.9 (1)	1.8 (2)	1.8 (2)	0.9 (1)
Lys	-	-	2.0 (2)	-	1.0 (1)	1.0 (1)	-
His	-	-	-	-	-	-	n.d.(3)
Arg	1.0 (1)	-	-	1.0 (1)	-	-	0.9 (1)
Trp	-	-	-	n.d.(1)	-	-	n.d.(1)
Total	(5)	(5)	(9)	(10)	(11)	(16)	(19)

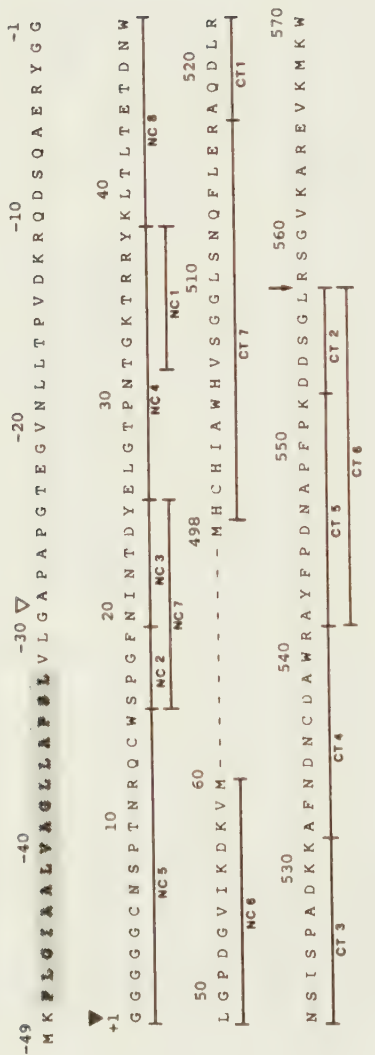


Fig. 22: Determination of the amino-terminus and the carboxyl-terminus of extracellular *N. crassa* laccase. Chymotryptic peptides (NC 1-8) isolated from the amino-terminal (CB-N) and tryptic peptides (CT 1-7) isolated from the carboxyl-terminal (CB-C) cyanogen bromide fragment are aligned to the amino acid sequence predicted by the nucleotide sequence of the gene (Fig. 15). The peptides were isolated by HPLC (see Figs. 20 and 21) and characterized by amino acid analysis as presented in Tables 8 and 9, respectively. The amino-terminus of the mature enzyme is marked by a solid triangle, the carboxyl-terminus is indicated by an arrow. The stippled area represents the hydrophobic core of the putative secretory signal sequence and the most probable leader cleavage site (von Heijne, 1986) is shown as an open triangle.

2. 8. Laccase biosynthesis in *N. crassa* wild type strain FGSC #321

Laccase was induced in rapidly growing 2-day old liquid cultures of *N. crassa* wild type strain FGSC #321 by adding cycloheximide at a concentration of 2.8 μ M (see section 2.1). The growth of the fungus and the amounts of laccase mRNA, intracellular and excreted laccase were followed as a function of time. When compared to control cultures growth of induced *N. crassa* cultures is inhibited by approximately 65% during the first 48 h after administration of cycloheximide (Fig. 23). About 100 h after inoculation autolysis starts both in induced and non-induced cultures, manifested by a decrease in cell mass. Already 72 h after inoculation the amounts of total cellular RNA begin to decrease slowly (Fig. 24, $t_{1/2} \sim 30$ h).

Laccase mRNA levels were measured by northern blot analysis of total cellular RNA and subsequent hybridization to a nick-translated laccase-specific DNA fragment. A single transcript with an estimated length of 2.5 kb was detected by this method (Fig. 25). As clearly shown in Fig. 26 expression of the laccase gene is turned on very rapidly after administration of cycloheximide. Laccase mRNA levels reach a saturation value within 30 - 40 min and stay nearly constant for about 48 h. Then laccase mRNA is degraded. Hence, only small mRNA species can be detected.

Intracellular laccase levels were estimated by SDS polyacrylamide gel electrophoresis of crude extracts, followed by western blotting and immunostaining with anti-laccase antiserum (Fig. 27). By this method two predominant bands with an apparent M_r of 63 - 66 kD and several minor bands with a higher M_r of up to 90 kD are detected. Between 2 and 12 h after addition of cycloheximide increasing amounts of intracellular laccase are observed. Then the levels remain more or less constant through the whole growth period examined. Intracellular laccase seems to present in an inactive form since no enzymic activity is found by assaying crude extracts.

Increasing amounts of extracellular laccase are detected immunologically in cultures grown for about 19 h in the presence of cycloheximide (Fig. 28). Low laccase activity can already be measured in the culture medium collected 12 h after induction. As shown in Fig. 23, laccase activity increases continuously reaching a maximal value 96 h after induction.

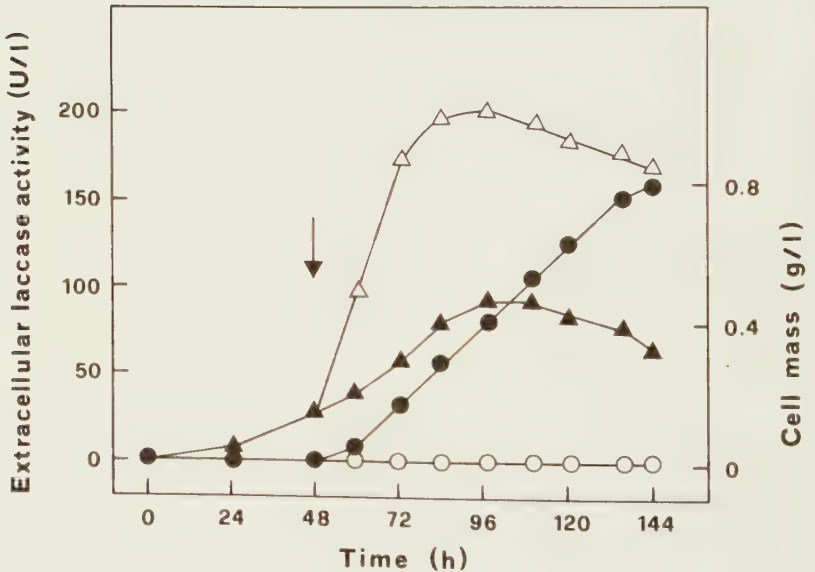


Fig. 23: Induction of extracellular laccase in *N. crassa* wild type strain FGSC #321. Cycloheximide was added 48 h after inoculation (arrow) at a concentration of 2.8 μ M. Growth (dry weight) of control (Δ) and induced ($\blacktriangle$) cultures and laccase activity in the corresponding culture medium (control, $\bigcirc$; induced, $\bullet$) were followed as a function of time.

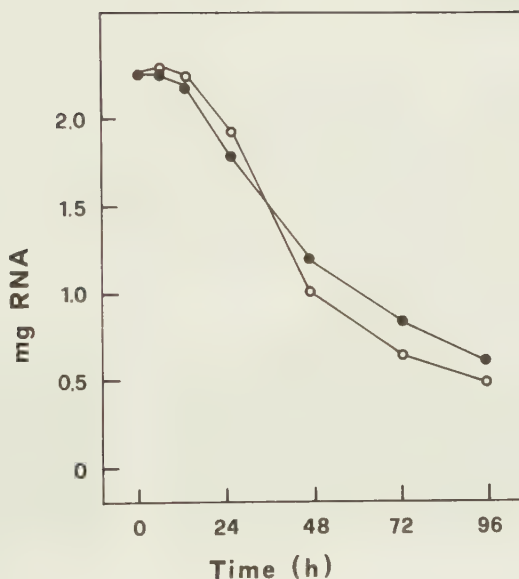


Fig. 24: Total cellular RNA levels in cycloheximide-induced (●) and non-induced (○) *N. crassa* cultures. RNA was prepared as described in materials and methods from 0.1 g mycelium and quantified by measuring absorbance at 260 nm (Maniatis et al., 1982). The values represent the average of three preparations.

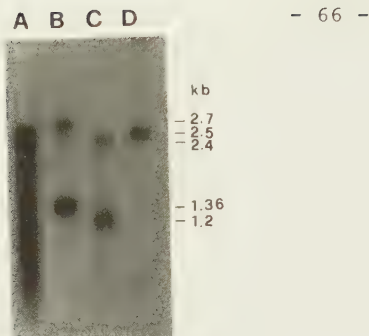


Fig. 25: Estimation of the length of laccase mRNA by Northern hybridization. 1.4 μ g poly(A)RNA isolated from cycloheximide-induced *N. crassa* cultures (lane A) and purified laccase specific DNA fragments (lanes B - D) were hybridized with the nick-translated 1.36 kb *Sal*I fragment (Fig. 8). The filter was washed at 68°C in 0.1 x SSC, 0.1 % (w/v) SDS. Experimental details are given in materials and methods. In lanes B - D purified DNA fragments labeled at their 5'ends were applied.

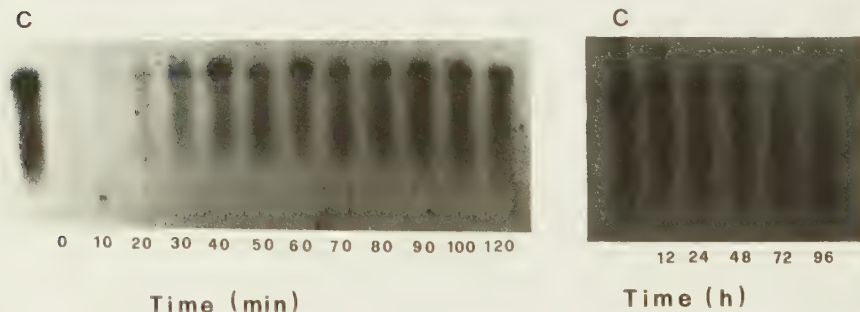


Fig. 26: Kinetics of laccase mRNA induction in *N. crassa* after addition of cycloheximide. mRNA was detected by Northern hybridization. 14 μ g of total cellular RNA were applied per lane and hybridized to a laccase-specific DNA fragment (see Fig. 25). The filters were washed at 68°C in 0.1 x SSC, 0.1 % (w/v) SDS. 'C' indicates a positive control (1.4 μ g poly(A)RNA, see Fig. 25, lane A).

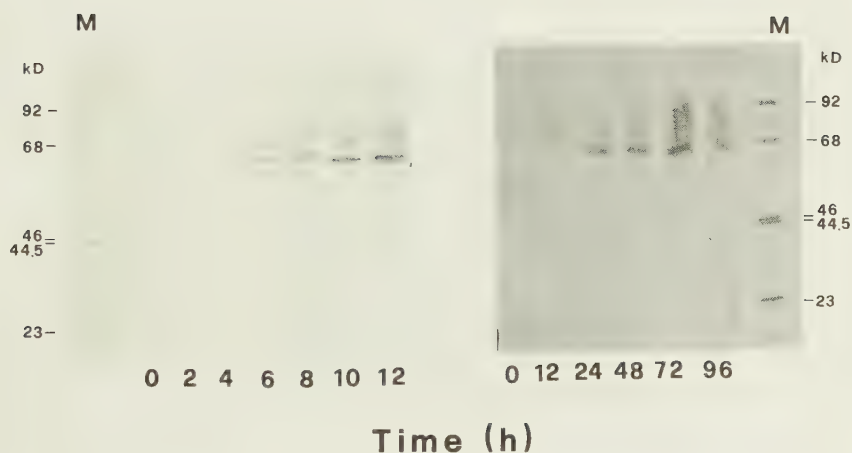


Fig. 27: increase of intracellular laccase levels after administration of cycloheximide to rapidly growing *N. crassa* cultures. Crude extracts were prepared from 0.1 g freeze-dried mycelia by homogenizing them with 3 ml 0.1 M sodium phosphate pH 6.0. The protein contents were estimated according to Lowry et al., (1951) using BSA as a standard. Equal amounts (30 µg) of intracellular proteins were applied to a 10 % (w/v) SDS polyacrylamide gel. Laccase was detected by immunoblotting as described in materials and methods.
'M' indicates a M_r marker.

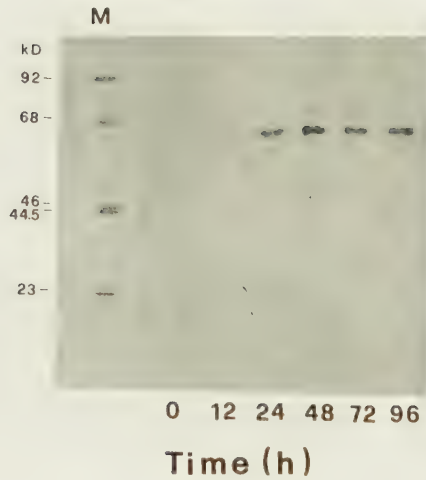


Fig. 28: Increase of extracellular laccase after administration of cycloheximide to rapidly growing *N. crassa* cultures. Laccase levels were detected by immunoblotting as described in materials and methods. The protein contents of 0.5 ml culture medium was applied per lane.
'M' indicates a M_r marker.

3. DISCUSSION

3. 1. Molecular cloning of the *N. crassa* laccase gene

Gene cloning provides a very straightforward way to determine the complete amino acid sequence of a large molecule such as laccase. From the small haploid genome (27'000 kbp; Krumlauf & Marzluf, 1979) of the filamentous fungus *N. crassa* several nuclear genes have been isolated by a variety of basic approaches. These include complementation of specific mutants of *N. crassa* (e.g. Atkins & Lambowitz, 1985) or *E. coli* (e.g. Vapnek *et al.*, 1977; Schechtman & Yanofsky, 1983; Buxton & Radford, 1983; Dunn-Coleman, 1984; Legerton & Yanofsky, 1985), screening of cDNA libraries by hybridization selection of mRNA, followed by cell-free protein synthesis and immunosorption (e.g. Arends & Sebald, 1984; Harnisch *et al.*, 1985), screening of cDNA expression libraries by using antibodies to specific proteins (e.g. Hager *et al.*, 1986), and screening of cDNA and/or genomic DNA libraries by hybridization with heterologous or homologous DNA fragments (e.g. Woudt *et al.*, 1983; Orbach *et al.*, 1986) or synthetic oligonucleotide probes (e.g. Kinnaird *et al.*, 1982; Munger *et al.*, 1985). Each of these methods has serious limitations that prevent its general utility. Although a large number of *N. crassa* mutants have been isolated and mapped, no strain specifically unable to produce laccase is available. Therefore, the approach involving complementation of mutants could not be applied to cloning of this gene. Antibodies against SDS-denatured *N. crassa* laccase, which recognized the *in vitro* translated product (see section 2.2.) could in principal have been used to screen a cDNA library. However, we decided to clone the *N. crassa* laccase gene by screening of a genomic DNA library with specific synthetic oligonucleotide probes for the following reasons:

1. Synthetic oligonucleotide primers provide a powerful tool for the efficient detection and transcription of specific mRNA species and thus have been often used to isolate cDNA or genomic DNA clones.

2. *N. crassa* laccase is an inducible enzyme, hence, high amounts of laccase mRNA were expected to be present during *de novo* biosynthesis of the protein.

3. By preliminary sequence analysis of the primary structure of *N. crassa* laccase a tryptic decapeptide was isolated which allowed to design a unique laccase-specific oligonucleotide probe.

4. Only genomic clones provide information about structural organizations of genes and promoter regions and allow the study of the regulation of gene expression.

The method described for the molecular cloning of the *N. crassa* laccase gene is rapid and very powerful. It was also used by our laboratory to clone two other nuclear genes of *N. crassa*, namely copper metallothionein (Münger *et al.*, 1985) and tyrosinase (Lerch, unpublished results).

Retrospectively, the primer extension was the most important and at the same time the most critical step of the isolation procedure. The part of the tryptic decapeptide (residues 5-8; Fig. 3A) which had been chosen to design a unique laccase-specific deoxyundecanucleotide offered eight different possibilities (Fig. 3B). The sequence of the 11-mer synthesized (Fig. 3C) was preferred to the other seven possibilities based on the reported codon usage in other nuclear *N. crassa* genes (Schechtman & Yanofsky, 1983; Woudt *et al.*, 1983; Kinnaird & Fincham, 1983). Sequence analysis of the corresponding part of the laccase gene (Fig. 9) revealed that the designed 11-mer used for primer extension obviously worked with one mismatch in the centre of the molecule. Hence, in agreement with studies of Romaniuk *et al.* (1979) G·U base pairing apparently does not markedly interfere in forming RNA-DNA duplexes. It was also demonstrated that cDNA synthesis is primed with a mixture of degenerated 17-mers (16 different species), however, sufficiently enriched mRNA is an absolute prerequisite (Lerch, unpublished results).

By sequence analysis of the specifically primed cDNA approximately 30 bases were determined unambiguously. These included the coding region

from the priming site towards the amino-terminus (Fig. 3D). Based on this information a unique 21-mer (Fig 3F) was synthesized which was used to screen a genomic *N. crassa* DNA library. The melting temperature of this hybridization probe was estimated to be 58° according to the 'Wallace' rule ($T_d = (2 \times \text{number of A-T bp}) + (4 \times \text{number of G-C bp})^\circ\text{C}$; Wallace *et al.*, 1979). As shown in Fig. 5B, positive clones could be clearly identified at 55° under low stringent washing conditions, but no hybridization signal was detected at 60°.

Approximately 11 genome equivalents (about 60'000 *E. coli* colonies containing *N. crassa* DNA inserts of an average length of 5 kb) were screened with the 21-mer and seven positive clones were isolated. This ratio indicates the occurrence of a single laccase gene in *N. crassa* and is also consistent with the finding of a single restriction fragment of a complete digest of genomic DNA hybridizing to a laccase-specific DNA probes (Fig. 10).

Unfortunately all five different positive clones obtained by screening of a genomic DNA library contained only part of the laccase gene (Fig. 6). Interestingly, they were all lacking the 5'end of the coding region of the laccase gene. The *N. crassa* DNA inserts ended at only two particular Sau3A sites, although four Sau3A sites are present within the complete structural gene. Hence, not every target in the DNA is recognized by the restriction enzyme with equal probability during partial digests of DNA.

3. 2. Structural characterization of the laccase gene of *N. crassa* wild type strain TS

3. 2. 1. The coding region

By comparing the amino acid sequence predicted from the nucleotide sequence with protein chemical data the coding region of the laccase gene of *N. crassa* wild type strain TS was deduced. The gene encodes a 619-amino-acid polypeptide with a M_r of 68'128. The reading frame is

interrupted by a short 57-bp intron near to its 5' end. The general guanine+cytosine (G+C) content of the structural gene including its 5' and 3' flanking regions is 56.9% which is slightly above the average value of 54% of the overall *N. crassa* genome (Villa & Storck, 1968). The G+C content of the coding part is 59.4% and significantly enhanced when compared to the 5' and 3' flanking regions (45.8% and 52.5%) or the intron (45.6%). The high G+C content of the coding region is due in part to the strong preference (76.2%) for codons ending in G or C.

3. 2. 2. The codon usage

In Table 10 the codon usage of the *N. crassa* laccase gene is compared to that of other nuclear *N. crassa* genes. The comparison confirms the hypothesis that the codon usage in *N. crassa* correlates with the level of gene expression (Kinnaird & Fincham, 1983; Orbach *et al.*, 1986). A similar dependence has been found in other organisms, i. e. *E. coli* (Ikemura, 1981) and *Saccharomyces cerevisiae* (Bennetzen & Hall, 1982). The codon usage in *N. crassa* genes that are virtually constitutively expressed at high levels (e.g. the H3 and H4 histone genes, the *am* gene and β -tubulin gene) is strikingly biased in the following way:

1. If there is a choice between purines and pyrimidines in the third positions of codons, the latter are strongly preferred.
2. If a codon is ending with a purine guanine is rather used than adenine.

As a consequence only 29 out of the 61 sense codons are used in the H3 and H4 histone genes, 39 in the *am* gene, and 42 in the β -tubulin gene. The genes that are subjected to strong regulation of different kinds, however, show much less bias and contain nearly all of the 61 possible codons (*trp*-1: 57; *his*-3: 58, laccase: 59).

Table 10: Codon usage in nuclear *N. crassa* genes

Amino acid	Codon	lac	trp-1	his-3	H-4	am	tub-2	Amino acid	Codon	lac	trp-1	his-3	H-4	am	tub-2
Phe	UUU	5	7	8	-	-	1	-	UCU	2	14	13	3	1	3
	UUC	14	15	21	4	2	17	25	UCC	5	20	14	6	1	21
Leu	UUA	-	-	-	-	-	-	-	UCA	2	1	4	-	-	-
	UUG	2	10	5	-	-	1	2	UGG	15	8	11	1	-	1
	CUU	4	21	23	1	1	13	6	AGU	2	4	5	-	-	-
	CUC	25	26	32	12	7	22	25	AGC	8	12	17	2	-	3
	CUA	1	2	1	-	-	1	-	CCU	11	11	11	-	-	3
Ile	CUG	20	18	15	1	-	1	1	CCC	13	20	29	5	1	10
	AUG	8	11	14	2	1	6	4	CCA	3	5	3	-	-	-
	AUC	26	33	31	5	6	12	9	CGG	15	4	6	-	-	-
	AUA	1	-	-	-	-	-	-	ACU	4	5	18	2	1	8
Met	AUG	10	15	6	1	1	9	20	ACC	23	22	23	6	8	17
Val	GUU	9	11	17	-	1	14	10	AGA	3	2	9	-	-	-
	GUC	15	39	41	6	6	19	23	AGC	10	5	2	-	-	-
	GUA	1	3	5	-	-	-	-	GGU	5	19	35	5	2	15
	GUG	15	13	7	-	-	-	3	GCC	22	37	50	8	5	36
Tyr	UUA	5	6	3	-	-	-	1	GCA	5	5	1	-	-	-
	UAC	18	10	11	3	4	15	13	GGG	10	7	5	-	-	1
His	CAU	6	5	6	-	1	-	4	UGU	2	-	3	-	-	1
	CAC	12	13	17	2	1	9	6	UGC	5	10	12	1	-	5
Gln	CAA	4	7	5	-	-	-	3	UGG	16	5	8	-	-	8
	CAG	19	23	27	9	2	15	19	CGU	2	12	20	7	8	10
Asn	AUU	9	4	4	-	-	1	1	CGC	7	12	10	10	6	9
	AAC	43	20	17	2	1	22	24	CGA	-	-	9	-	-	-
Lys	AAA	4	1	4	-	-	-	-	CGG	11	3	7	-	-	-
	AAG	23	43	49	14	11	29	13	AGA	4	4	3	-	-	-
Asp	GAU	8	16	15	-	1	3	9	AGG	3	6	9	-	-	2
	GAC	33	24	22	4	2	12	12	GGU	10	18	17	4	9	31
Glu	GNA	2	3	5	-	-	-	1	GGC	36	34	26	3	6	23
	GAG	10	48	54	7	4	36	38	GGA	8	7	8	-	2	-
									GGG	5	3	-	-	-	1

Abbreviations of genes and references see Table 12

3. 2. 3. The intron

The region coding for the amino-terminal part of *N. crassa* laccase is interrupted by a short intron. It is striking that most introns defined so far are usually positioned much nearer to the 5'end of nuclear *N. crassa* genes (e.g. Kinnaird & Fincham, 1983; Arends & Sebald, 1984; Hager *et al.*, 1986; Orbach *et al.*, 1986). The intron of the laccase gene has a small size (57) comparable to many other intervening sequences in *N. crassa* (Table 11). Moreover it shares conserved 5' and 3'splicing sites and a conserved internal sequence with other *N. crassa* introns. The *N. crassa* consensus splice sequences are similar to the *S. cerevisiae* consensus signals probably indicating analogies in splicing mechanisms. This hypothesis has recently been tested by studying removal of a *N. crassa* intron of the histone H3 gene in yeast (Woudt *et al.*, 1985). Although the intron was correctly removed, the efficiency was low since the internal element was not perfectly conserved (TGCTAAC instead of TACTAAC). Moreover the intron was only removed when it was located near to the 5'end of the primary transcript.

3. 2. 4. The transcription initiation site

Three transcription startpoints were detected by nuclease S1 mapping of the region flanking the coding part of the laccase gene on the 5'side. The major transcription initiation site is located at a distance of 193 bases and two minor ones are found 192 and 118 bases upstream of the initiation codon. It has been shown that the length of 5'untranslated regions in nuclear *N. crassa* genes is very heterogeneous and ranges from 46 (Arends & Sebald, 1984) up to 405 bases (Giles *et al.*, 1985). The sequence CTTCA of the major transcription site in the laccase gene is similar to those of the ADP/ATP carrier gene (CTCCA, Arends & Sebald, 1984), the *am* gene (GCTCA, Kinnaird & Fincham, 1983), and the copper metallothionein gene (CACTA, Munger *et al.*, 1985). Furthermore in all these genes a typical TATA box (Breathnach & Chambon, 1981) is positioned 45 - 56 bases upstream of the transcription initiation sites and

Table 11: Introns in N. crassa genes

Gene	No.	Length (bp)	5'Site	Internal consensus	3'Site
am	1	66	GTACGT	AGCTGACT-----16-----	CAG
am	2	61	GTAAGT	TGCTGACT-----12-----	CAG
acp-1	1	280	GTATGT	TGCTAACA-----14-----	CAG
acp-1	2	192	GTACGT	TGCTGACG-----6-----	TAG
fes-1	1	323	GTGAGT	AGCTGACG-----16-----	CAG
fes-1	2	121	GTAAGG	TGCTGACC-----10-----	CAG
fes-1	3	121	GTACGC	GACTGACA-----18-----	CAG
hat-1	1	53	GTAAGT	TACTAACC-----7-----	TAG
hat-1	2	124	GTACGT	TGCTGACT-----10-----	CAG
hat-1	3	64	GTAGCT	CGCTAACC-----11-----	CAG
hat-1	4	67	GTAAGT	AGCTAACC-----12-----	CAG
his-3		59	GTAAGT	CTTTAACT-----9-----	TAG
H-3		67	GTAAGT	TGCTAACT-----13-----	CAG
H-4	1	68	GTAAGT	GACTGACT-----16-----	CAG
H-4	2	69	GTACGT	AATAACA-----16-----	CAG
tub-2	1	240	GTAAGT	CGCTGACA-----18-----	TAG
tub-2	2	74	GTAAGT	GACTAACA-----13-----	CAG
tub-2	3	68	GTACGT	CGCTGACA-----7-----	CAG
tub-2	4	65	GTACGT	AGCTCACC-----8-----	CAG
tub-2	5	73	GTGAGC	CGCTAACT-----14-----	CAG
tub-2	6	57	GTAAGT	TACTGACG-----7-----	TAG
CuMT		94	GTAAGT	TACTAACC-----12-----	TAC
lac TS		57	GTAAGT	GACTGACG-----7-----	TAC
lac OR		57	GTAAGT	GACTGACA-----7-----	TAC
<u>N. crassa</u> consensus			GTA ^A _C GT	N ^A _G CT ^A _G ACN-----7 - 18-----	C _T AG
Yeast consensus ¹			GTATGT	TACTAACA-----18 - 53-----	C _T AG
Higher eucaryote ² consensus			GTAAGT		CAG

References: 1: Langford et al., 1984

2: Lerner et al., 1980

Abbreviations of N. crassa genes and references see
Table 12

they all lack a second element, the CAAT box (Breathnach & Chambon, 1981), which is usually present in most eucaryotic promoters in the -100 region.

3. 2. 5. The polyadenylation site

The polyadenylation signal AATAAA (Proudfoot & Brownlee, 1976), typical for higher eucaryotes, has so far been detected only in the histone H3 gene of *N. crassa* (Woudt *et al.*, 1983). The length of the 3' non-translated regions varies considerably and no consensus sequences characterizing a polyadenylation signal or the transcription termination site have so far been suggested. The polyadenylation site of the *N. crassa* laccase gene is located 281 bases downstream of the termination codon and is particularly rich in thymidine and cytosine.

3. 3. Laccase: an example of polymorphism in *N. crassa*

The laccase gene was isolated from two unrelated *N. crassa* wild type strains. Both were collected in nature in the U.S.A. Wild type strain TS (69-1113) was used by Horowitz and coworkers (Horowitz *et al.*, 1961, 1970a) to study the regulation of tyrosinase synthesis. This strain produces a thermostable form of tyrosinase with known primary structure (Rüegg *et al.*, 1982). Moreover, induction of laccase was studied in this strain (Froehner & Eriksson, 1974a) and some properties of the isolated enzyme have been reported (Froehner & Eriksson, 1974b; Lerch *et al.*, 1978). Wild type strain OR (74-OR8-1a) was used to generate a genomic *N. crassa* DNA library (Schechtman & Yanofsky, 1983), allowing the molecular cloning of several nuclear genes (e.g. *trp-1*). The asexual development of wild type strain OR (opposite mating type 74-OR23-1A) was investigated both at transcriptional (Berlin & Yanofsky, 1985b) and translational (Berlin & Yanofsky, 1985a) level. Moreover the strain was found to be able to produce extracellular laccase (data not shown).

Nucleotide sequencing of the laccase genes of the two *N. crassa* wild type strains TS and OR revealed the presence of two different allelic forms (Fig. 14). Considering the laccase genes from the transcription initiation to the polyadenylation sites an overall divergence of 5.3% is manifested as a result of 16 deletions, 13 insertions and 108 substitutions of bases. The point mutations include an unusually high percentage (40%) of transversions. The frequency of changes in non-translated regions is much larger than that of the coding regions. The divergence of the 5'non-translated leader is 18.8%, of the intron 8.7% and of the 3'trailer 10.8%, compared to 2.6% in the exons. Mutations in the non-translated regions, however, are potentially neutral since they do not affect regulatory signals such as the TATAAA box, the transcription initiation site, the conserved sequences of the intron or the polyadenylation site.

The two different laccase genes of *N. crassa* wild type strains OR and TS conform to the same structural organization, i. e. the length of the reading frame and the intron-exon boundaries are conserved. Heterogeneity in the coding regions is manifested by 31 changes in silent sites and 19 in replacement sites. As a result, the encoded protein chains differ in 12 out of 619 amino acid residues (Table 5) and thus show a divergence of 1.94%. Interestingly, the amino acid sequence variations are not distributed equally over the whole primary structures of the two isoenzymes. The amino-terminal region up to Trp 48, the region between Asp 64 and Leu 242 as well as the carboxyl-terminal part starting at Thr 391 are highly conserved. The constancy in structure may reflect its importance to the functional activity of laccase. In contrast, a shorter stretch of 88 amino acids (residues 243-330) with a divergence of 10% may represent a segment with less functional importance. It is striking to note a cluster of amino acid substitutions formed in a small region between residues 297 and 306. It may be assumed that the polymorphism of laccase in *N. crassa* is stable and neither allelic form has any relative advantages. The isoenzymes do not differ functionally and display the same physicochemical properties (e.g. pI-value, hydropathy profile, data not shown). It is tempting to speculate that more than the two described allelic forms of laccase occur in natural strains of *N. crassa*.

Most interesting, tyrosinase represents another example of polymorphism in *N. crassa*. Two allelic forms, distinguishable on the basis of their thermostability, were detected by Horowitz & Fling (1953). The primary structures of tyrosinase isolated from three different wild type strains were investigated by Rüegg *et al.* (1982). Similarly to laccase a high number of substitutions was found between the isoenzymes, although they showed the same catalytical properties. Hence, both tyrosinase and laccase are tolerant towards a high degree of variability without becoming functionally impaired. The two enzymes are not involved in the primary metabolism of *N. crassa* and therefore are not essential for the growth of the fungus. Consistent with the view of Wilson *et al.* (1977) the polymorphisms of both tyrosinase and laccase may be ascribed to the weakness of the selective forces acting on dispensable proteins.

3. 4. Posttranslational processing of *N. crassa* laccase

The translation product predicted by the laccase gene of *N. crassa* (wild type strain TS) is a protein with a molecular mass of 68'128 daltons, indicating that the size of laccase estimated by *in vitro* translation analysis (61 kD) is apparently too low. The nucleotides preceding the initiator-methionine conform to the consensus sequence found in nuclear genes of *N. crassa* (Table 12). Protein chemical studies revealed that laccase is synthesized as a precursor which exceeds the mature extracellular enzyme by 49 amino acids at its amino-terminal and 13 amino acids at its carboxyl-terminal end. As discussed below the amino-terminal extension may be required for secretion of the protein, whereas the carboxyl-terminal processing may be important for activation of the enzyme.

The amino acid composition (Table 13) derived from the amino acid sequence of the mature laccase (Fig. 15) is in good agreement with those determined earlier by amino acid analysis of the isolated protein (Froehner & Eriksson, 1974b; Lerch *et al.*, 1978). Extracellular laccase was furthermore shown to be a glycoprotein with a carbohydrate content of 11-12%. Indeed seven potential N-glycosylation sites Asn X Ser/Thr

Table 12: Sequences preceding the initiation-codon in nuclear N. crassa genes

Gene	Nucleotide sequence
am	TCAAA ATG
acp-1	TCACA ATG
fes-1	ACACA ATG
hat-1	CGCCA ATG
his-3	ACACC ATG
H-3	TCACA ATG
H-4	TCAAA ATG
trp-1	TCACA ATG
tub-2	TCAAG ATG
CuMT	TCAAA ATG
lac	TCACC ATG
Consensus:	TCA ^{AA} _{CC} ATG

- am: NADP dependent glutamate dehydrogenase; Kinnaird & Fincham, 1983
- acp-1: ATP/ADP carrier protein; Arends & Sebald, 1984
- fes-1: Iron sulphur subunit of ubiquinol-cytochrome C reductase; Harnish et al., 1985
- hat-1: Plasma membrane H⁺ATPase; Hager et al., 1986
- his-3: Histidine biosynthetic pathway multifunctional polypeptide; Legerton & Yanofsky, 1985
- H-3: Histone H3; Woudt et al., 1983
- H-4: Histone H4; Woudt et al., 1983
- trp-1: Tryptophan biosynthetic pathway trifunctional polypeptide; Schechtman & Yanofsky, 1983
- tub-2: β -tubulin; Orbach et al., 1986
- CuMT: Copper metallothionein, Munger et al., 1985
- lac: Laccase

Table 13: Amino acid composition of extracellular laccase of N. crassa wild type strain TS

Amino acid	1	2	3	%
Ala	37.8	35.7	34	6.1
Arg	22.7	23.4	23	4.1
Asx	83.3	87.0	90	
Asn			51	9.1
Asp			39	7.0
Cys	2.1	6.6	7	1.3
Glx	37.8	32.3	30	
Gln			21	3.8
Glu			9	1.6
Gly	51.9	51.3	51	9.3
His	17.2	15.6	18	3.2
Ile	29.1	33.5	34	6.1
Leu	41.1	43.5	44	7.9
Lys	21.5	22.3	22	3.9
Met	6.4	8.8	8	1.4
Phe	20.4	15.6	18	3.2
Pro	35.7	35.7	38	6.8
Ser	29.1	31.2	31	5.6
Thr	36.7	37.9	38	6.8
Trp	28.1	17.7	15	2.7
Tyr	21.6	24.4	22	3.9
Val	34.5	34.5	34	6.1
Total	557.0	557.0	557	100

References: 1: Froehner & Eriksson, 1974b
 2: Lerch et al., 1978
 3: Composition according to sequence analysis (Fig. 15)

Values of references 1 and 2 were normalized to 557 amino acid residues.

(Kornfeld & Kornfeld, 1985) are present in its primary structure located at positions 90, 233, 246, 291, 373, 395, and 469.

N. crassa laccase functions as an extracellular enzyme. Most secretory proteins are synthesized as precursors containing a transient amino-terminal signal sequence which serves to initiate translocation across membranes. Upon export this signal sequence is cleaved from the mature protein. Despite a great variability in terms of overall length and amino acid composition, three structurally (and possibly functionally) distinct regions have been recognized as the basic elements of a secretory signal sequence: a positively charged amino-terminal region, a central hydrophobic core, and a more polar carboxyl-terminal region that seems to define the cleavage site (Perlman & Halvorson, 1983; von Heijne, 1983). The amino-terminal portion of the protein which does not appear in mature extracellular *N. crassa* laccase exhibits most characteristics of a signal peptide. A positively charged residue (Lys -48) precedes a hydrophobic core of approximately 16 amino acids (Fig. 22). A hydropathy analysis (Fig. 29) revealed that the putative signal sequence is the only region of laccase demonstrating a pronounced hydrophobic character. The remaining segment of the postcore is much longer than the usually observed 1 - 5 residues. Seven out of the 31 amino acids are charged and a very hydrophilic character is indicated. The model signal-sequence cleavage site (i.e. a small residue (Ala, Ser, Gly, Cys, Thr, or Gln) in position -1 and absence of an aromatic, charged or large and polar residue in position -3) is not evident. The most likely signal cleavage site predicted by an empirical method (von Heijne, 1986) is found between residues -29 and -28. Most interesting the amino-terminal presequence of laccase shares similar properties with those of two other extracellular enzymes isolated from fungi (cutinase; Soliday *et al.*, 1984) and *Dictyostelium* amoebae (cyclic nucleotide phosphodiesterase; Lacombe *et al.*, 1986). In all these three examples primary processing would not be expected to occur at the amino-terminal residue of the purified extracellular protein. Hence, primary processing may be suggested to involve an unusual mechanism, but a putative secondary processing can not be ruled out either.

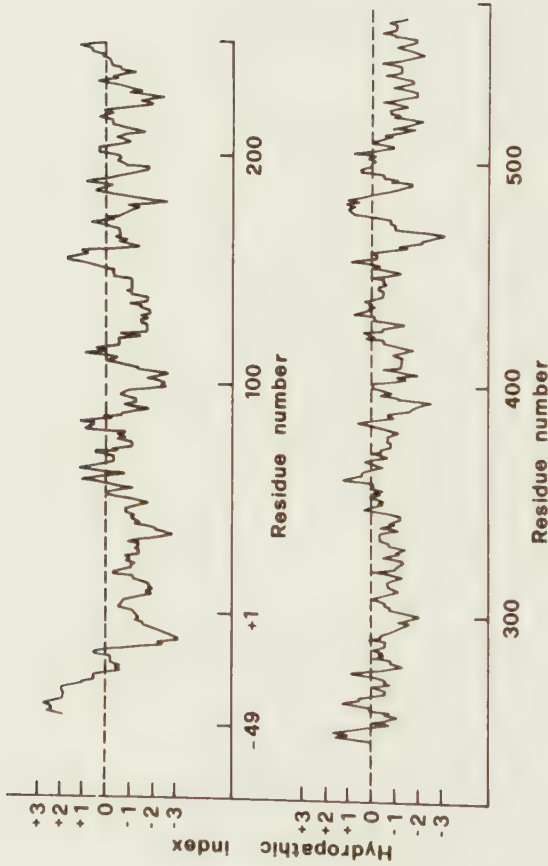


Fig. 29: Hydropathy profile of *N. crassa* laccase. The amino acid sequence was deduced from the nucleotide sequence of the gene (Fig. 15). Numbering starts at the amino-terminus of the mature enzyme (Fig. 22). Hydropobicity values were calculated using a window size of 9 residues according to Kyte and Doolittle (1982). Values indicating hydrophobic and hydrophilic regions are above and below zero, respectively.

Analysis of the carboxyl-terminus of purified *N. crassa* laccase revealed the unexpected finding of a carboxyl-terminal processing of the translation product. The carboxyl-terminal trailer peptide of laccase is relatively short (13 amino acids) and has a conspicuously basic character (three Lys; two Arg). Its pI-value is estimated according to Pustell & Kafatos (1984) to be 11.2, a value considerably higher than those of the mature enzyme (6.83) and the amino-terminal prepeptide (7.00). The biological significance of the carboxyl-terminal processing step during maturation of laccase is unknown. It is also unclear whether the carboxyl-terminal elongated protein shows laccase activity or not. However, due to the outstanding basic character of the trailer peptide an inhibitory effect must be considered. Thus the formation of a loop and interaction with the active site may prevent binding of copper ions. A direct assessment of the importance and the role of the carboxyl-terminal extension, however, will only come from specific modifications of its coding region.

Interestingly a second *N. crassa* phenoloxidase, namely tyrosinase, was also demonstrated to be derived from a precursor with carboxyl-terminal elongation (Lerch, unpublished data). Moreover the two mature enzymes end with a Leu-residue (Laccase) and a Phe-residue (tyrosinase), which could easily constitute a cleavage site for a protease with chymotrypsin-like activity. Considering the coinduction in vegetative cultures of *N. crassa* (see section 1. 4.), it may be proposed that activation of the two enzymes is controlled by a common endopeptidase.

Taken together, the data indicate a complex maturation pathway of extracellular *N. crassa* laccase. Whereas amino-terminal processing and glycosylation may be required for secretion, carboxyl-terminal processing may play a role in activation of the enzyme.

3. 5. Amino acid sequence comparison of *N. crassa* laccase with other blue copper proteins

An amino acid sequence homology search revealed that only the carboxyl-terminal region of *N. crassa* laccase is closely related to other blue copper proteins. In Fig. 30 part of the amino acid sequence of *N. crassa* laccase is compared with homologous parts of human ceruloplasmin (Takahashi *et al.*, 1984) and with the primary structures of plastocyanin from *Populus nigra* var. *italica* (Ambler, 1980) and azurin from *Pseudomonas aeruginosa* (Ambler, 1971). Ceruloplasmin is thus far the only multicopper oxidase with completely known primary structure. The single polypeptide chain consists of 1046 amino acid residues and exhibits a 3-fold internal homology in amino acid sequence (Takahashi *et al.*, 1983; Ortel *et al.*, 1984). The protein binds seven copper ions per molecule namely two type 1, one type 2 and four type 3 (Table 1). Plastocyanin and azurin are low molecular weight blue copper proteins binding only one type 1 copper ion per molecule. Amino acid sequences of azurin and plastocyanin from a wide variety of sources have been determined (Adman, 1985; Rydén, 1984). Both poplar plastocyanin and azurin from *P. aeruginosa* have been crystallized and their three dimensional structures as well as their copper ligands are known (Colman *et al.*, 1978; Adman *et al.*, 1978). Plastocyanin and azurin have been shown to be divergently related (Rydén & Lundgren, 1976) and in several reports it has been suggested that the small blue copper proteins and the multicopper oxidases share a common ancestry (Kingston *et al.*, 1977; Dwulet & Putnam, 1981; Rydén, 1982). The amino acid sequence comparison shown in Fig. 30 further supports this hypothesis. The primary structure of *N. crassa* laccase is highly homologous to the carboxyl-terminal part of ceruloplasmin (CpC) and to a smaller extent to the other two homology units (CpN, CpM) of ceruloplasmin and to plastocyanin and azurin. In all six polypeptides compared five amino acid residues are invariant (one Cys, two Gly, two His) and four are isofunctional having a conserved aromatic (three Phe, Trp or Tyr) or hydrophobic (one Leu or Met) character. Among the invariant ones three represent ligands of the type 1 copper in plastocyanin (His 37, Cys 84 and His 87) and azurin (His 46, Cys 112 and His 117).

The fourth blue copper ligand in plastocyanin and azurin is Met 92 and Met 121, respectively. The corresponding residues Met 690 and Met 1031 are found only in CpM and CpC and represent the putative fourth binding sites for the two type 1 copper ions of human ceruloplasmin (Ortel *et al.*, 1984). Surprisingly, however, *N. crassa* laccase lacks a Met-residue at the homologous position. Hence, this finding implicates a type 1 copper coordination different from plastocyanin and azurin, and possibly from ceruloplasmin. In this context it is interesting to note that stellacyanin, a small blue copper protein isolated from *Rhus vernicifera* (Peisach *et al.*, 1967) is also devoid of methionine. The nature of its fourth copper ligand is presently still a matter of debate (Blair *et al.*, 1985).

As mentioned above the highest overall sequence homology is found when *N. crassa* laccase is compared with the carboxyl-terminal region of human ceruloplasmin. Thus, 114 amino acid residues of the third homology unit (CpC) of ceruloplasmin can be aligned with part of the primary structure of laccase to give 27 identical and 12 isofunctional amino acid residues (Fig. 30). Most interestingly, two short stretches rich in His show extensive sequence homology. A computer analysis using the unitary matrix indicated maximal homology scores of 6 and 9 for the segments presented in Figs. 31A and 31B, respectively (N. Takahashi and F. W. Putnam, personal communication). The remarkable similarity of these two regions suggests that they play an important role in the formation of the active site of multicopper oxidases. In particular, they could provide ligands to all three different types of copper in both enzymes. This suggestion is consistent with the observation of Raju (1983) that 50% of the non-blue copper of the ceruloplasmin molecule reside in the carboxyl-terminal tryptic peptide of 11 kD. As pointed out above at least three invariant type 1 copper binding residues are present within these two stretches. The fourth type 1 copper ligand in *N. crassa* laccase is currently not known (see above). It is conceivable, however, that the nearby Met 169 of *N. crassa* laccase is involved in copper binding. The idea that also type 2 or type 3 copper ligands could be provided by the two homologous regions shown in Fig. 31 is based on earlier reports. The sequence containing the His-cluster of ceruloplasmin (Fig. 31A) has been shown to be homologous

(next page)

Fig. 30: Amino acid sequence comparison between part of N. crassa laccase (Lac) and homologous parts of other blue copper proteins: Cp, human ceruloplasmin (Takanashi et al., 1984); CpN, first homology unit residues 234-344; CpM, second homology unit residues 595-705; CpC, third homology unit residues 933-1046; Pc, poplar plastocyanin (Ambler, 1980); Az, P. aeruginosa azurin (Ambler, 1971). The three homology units CpN, CpM, and CpC of ceruloplasmin are aligned as described by Takahashi et al. (1983). CpC and Pc are aligned according to Rydén (1982) and Pc and Az are aligned according to Murata et al. (1982) with some minor modifications maximizing the sequence homology of each primary structure to that of N. crassa laccase. The positions of the four copper ligands in Pc (Colman et al., 1978) and Az (Adman et al., 1978) are marked by triangles. The number on the left of each sequence identifies the position within the protein of the first amino acid residue shown. For Lac, the numbers refer to valine as 1 according to Fig. 9. Dashes represent gaps introduced for alignment. Boxes relate amino acid residues of Lac with identical residues in CpN, CpM, CpC, Pc, and/or Az. Shaded areas relate isofunctional residues (D and E; K and R; T and S; F, W, and Y; I, L, M, and V).

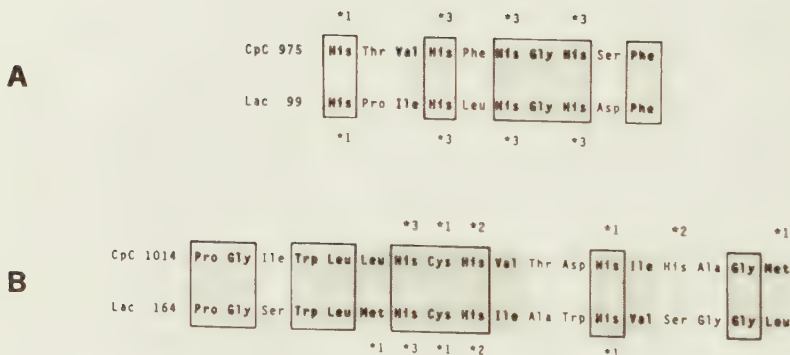


Fig. 31: Amino acid sequence comparison of two highly homologous regions (A and B) of *N. crassa* laccase (Lac) and the carboxyl-terminal homology unit (CpC) of human ceruloplasmin (Takahashi et al., 1983). Numbers on the left of each sequence identify the positions within the protein of the first residue shown. For Lac, numbers refer to valine as 1 according to Fig. 9. Identical amino acid residues are boxed and isofunctional amino acid residues are shaded (see Fig. 30). Potential ligands to the three different types of copper are indicated by *1, *2, and *3, respectively. The assignment of the copper ligands for *N. crassa* laccase is based on that proposed earlier for CpC (Rydén, 1982).

to that providing ligands to the non-blue copper site in Cu/Zn-superoxide dismutase (Ortel *et al.*, 1984). Furthermore, using the coordinates of the known three-dimensional structure of poplar plastocyanin (Adman *et al.*, 1978), Rydén (1982) recently presented a three-dimensional model of the copper active site of human ceruloplasmin. The model involves the carboxyl-terminal region (amino acid residues 932-1040, Takahashi *et al.*, 1984) and proposes His 975, Cys 1021, His 1026 and Met 1031 as type 1, His 1022 and His 1028 as type 2 and His 978, His 980, His 982 and His 1020 as type 3 copper ligands. The amino acid sequence comparison of ceruloplasmin with laccase supports this model with respect to the type 1 and the type 3 copper ligands. However, no His-residue is found in laccase that is homologous to the suggested type 2 copper binding His 1028 of ceruloplasmin. Hence, our data imply that either the structures of the type 2 site in ceruloplasmin and laccase are different or the homologous type 2 ligands are closer to the amino-terminus than suggested for ceruloplasmin. It is clear that the complete amino acid sequences of other multicopper oxidases are required to fully define the amino acid residues involved in the binding of the active site copper.

3. 6. Induction of laccase biosynthesis in cycloheximide-induced vegetative cultures of *N. crassa*

Exposure of rapidly growing vegetative cultures of *N. crassa* to small amounts of cycloheximide leads to *de novo* biosynthesis of extracellular laccase. Earlier studies of the induction of laccase in this fungus (Froehner & Eriksson, 1974a) led to the assumption that derepression of the molecule occurs on the transcriptional rather than the translational level. In order to test this hypothesis the abundance of laccase mRNA, as well as the amounts of intracellular and extracellular laccase were examined at different times using the described conditions for induction. Similar growth curves and kinetics of extracellular laccase production were obtained for two *N. crassa* wild type strains investigated (TS, Fig. 1; FGSC #321, Fig. 23). These results are also consistent with the data reported by Froehner & Eriksson (1974a).

Laccase-specific DNA probes detected a single mRNA species of about 2.5 kb which is in good agreement with the length of the transcript determined by primary structure analysis of the gene. Surprisingly a dramatic increase of laccase mRNA is observed only within the short time of 30-40 min after administration of cycloheximide (Fig. 26), indicating a very rapid derepression of the gene. About 2 h after induction increasing amounts of intracellular laccase are detected. The correlation of the increase of laccase mRNA with that of intracellular laccase suggests that regulation on the transcriptional level plays a major role in *N. crassa* laccase biosynthesis. Most interestingly also high levels of tyrosinase mRNA are observed within the first 30 min after induction with cycloheximide (data not shown). Moreover, studies using D-Phe as inducing agent also revealed a concomitant increase in synthesis of laccase and tyrosinase mRNA (data not shown). Hence, these results further corroborate the hypothesis that the two enzymes are controlled by a common regulatory mechanism (see 1. 4.). A promoter analysis of the two genes could demonstrate if homologous regulatory elements are located in the regions upstream of the transcription initiation sites. It would also be of interest to determine the chromosomal location of the two genes and to see whether they are present on the same linkage group in a clustered arrangement.

Both in cycloheximide-induced and non-induced *N. crassa* cultures the total amounts of cellular RNA start to decrease slowly 72 h after inoculation (Fig. 24). At this time the cultures are in the stationary growth phase having ceased to produce cell mass (Fig. 23). Laccase mRNA levels estimated by Northern analysis remain nearly constant for about 48 h after addition of cycloheximide (Fig. 26). Therefore degradation of laccase mRNA shows a delay of 24 h compared to the overall RNA degradation process. These data suggest that induction of *N. crassa* laccase is not only due to a high increase in the rate of transcription, but probably is also a result of mRNA stabilization.

Between 2 and 12 h after addition of cycloheximide *N. crassa* produces increasing amounts of intracellular laccase (Fig. 27). The lag period of approximately 1.5 h compared to the increase of mRNA could be explained

by the time needed for glycosylation and/or copper loading. Most secreted proteins whose biosynthetic processes have been analyzed were shown to be glycosylated co-translationally during the growth of the nascent polypeptide chain in the rough endoplasmic reticulum (Struck & Lennarz, 1977; Czichi & Lennarz, 1977). Moreover, intracellular laccase has earlier been demonstrated to exist as a glycoprotein by its ability to bind to concanavalin A sepharose (Froehner & Eriksson, 1974b). A very heterogeneous mixture of proteins is detected by SDS polyacrylamide gel electrophoresis, followed by Western-blotting and immunostaining with anti-laccase antiserum. Although we did not test if the different bands disappear by competition with native laccase, we suppose all of them to represent various forms of laccase developed during maturation. The two major bands with an apparent M_r of 63 - 66 kD are suggested to be partially or fully truncated laccase (see 3. 4.). The differences in size of the minor high M_r bands which manifest themselves by immunostaining of both intracellular and extracellular laccase may be the result of variations in carbohydrate content. This view is consistent with the heterogeneity observed when purified extracellular laccase is analyzed by SDS polyacrylamide gel electrophoresis, followed by protein- and carbohydrate staining (data not shown). Pulse and pulse-chase labeling experiments are necessary to correctly assign the variety of bands and to reveal their order of appearance. Moreover, investigations with tunicamycin, an antibiotic that specifically blocks the transfer of mannose- and glucosamine-rich carbohydrate-complexes from dolichol-phosphate carriers to proteins (Takasuki *et al.*, 1975; Tkacz & Lampen, 1975) in the rough endoplasmic reticulum (Struck & Lennarz, 1977), may provide more insight into the glycosylation process and the mechanism of secretion.

Approximately 12 - 19 h after addition of cycloheximide increasing amounts of extracellular laccase are detected both immunologically and by measuring activity (Figs. 28 and 23). The lag period of more than 10 h with respect to the appearance of intracellular laccase may be caused by copper loading and/or additional processing steps (e.g. secondary processing at the amino-terminal end and/or carboxyl-terminal processing; see 3. 4.).

In conclusion the presented data suggest that laccase biosynthesis in *N. crassa* is regulated primarily at the transcriptional level by a dramatic increase in transcription and mRNA stabilization. However, also regulation on the translational level is indicated by a complex pathway of maturation. Further investigations of the synthesis, intracellular processing, and secretion of laccase are necessary to elucidate the system(s) that control laccase biosynthesis in *N. crassa*. Moreover, additional studies of the regulation of gene expression in vegetative *N. crassa* cultures using other inducing agents (e.g. actinomycin D, puromycin, ethionine) or in cultures undergoing sexual differentiation may reveal the thus far unknown biological function of *N. crassa* laccase.

4. MATERIALS AND METHODS

4. 1. MICROBIOLOGICAL METHODS

4. 1. 1. General techniques and materials

Culture media and plasticware were sterilized by autoclaving (121°C, 20 min). Glassware was baked at 180°C for 2 h. Nutrients for the preparation of culture media were obtained from Difco, cycloheximide was from Sigma, ampicillin and chloramphenicol were purchased from Fluka. All other chemicals were of the highest grade of purity available and only doubly distilled water was used.

4. 1. 2. *Neurospora crassa*

1. Fungal strains

The *N. crassa* laccase gene was isolated from the wild type strain OR (74-OR8-1a, FGSC #988, Fungal Genetics Stock Center, Kansas City, KS, USA) and from the wild type strain TS (69-1113, donated by N. H. Horowitz, Pasadena, CA, USA). The regulation of the laccase gene was studied in the wild type strain TS which is FGSC #321 from the Fungal Genetics Stock Center (Kansas City, KS, USA).

2. Culturing conditions

All strains were maintained on agar slants according to Horowitz *et al.* (1970a).

For induction experiments strain TS was grown as described by Froehner & Eriksson (1974a) in Fries minimal medium containing 20% of the normal sulfur concentration (Horowitz & Shen, 1952). 0.8 l culture medium in 2 l

Erlenmeyer flasks (with intrusions) were inoculated with 10 ml of a conidial suspension in water (Absorption at 600 nm 0.4) and incubated at 35°C in the dark. After 48 h cycloheximide (8.5 mM in water) was added at a concentration of 2.8 μ M to induce laccase biosynthesis. The cultures were then incubated on a rotatory shaker (110 rpm) at 30°C in the dark for the times indicated.

The other wild type strains were grown in Vogel medium (Vogel, 1956). Immediately after inoculation the cultures were agitated at 25°C in the dark.

Mycelia were harvested by filtration through double layers of cheese cloth, washed with water, lyophilized and stored at -20°C.

4. 1. 3. *Escherichia coli*

1. Bacterial strains

The *E. coli* strains used in this study have the following genotypes:

E. coli LE392:

F⁻, hsdR514 (r_k⁻, m_k⁻) supE44, supF58, lacY1 or Δ (lacIZY)6, galK2, galT22, metB1, trpR55, λ ⁻ (Maniatis *et al.*, 1982).

E. coli JM83:

ara, Δ (lac, pro) strA, thi, (Φ 80), Δ lacZ Δ M15) (Vieira & Messing, 1982).

E. coli JM101:

Δ (lac, pro) thi, supE, F'tra D36, proAB, lac I^q, Z Δ M15 (Messing, 1983).

E. coli W.71.18:

Δ (lac, pro, rps¹) F' (lac I^q, lacZ, Δ M15, proB⁺, proA⁺ (Messing *et al.*, 1981).

Although the strain *E. coli* LE392 is commonly used to propagate bacteriophage λ vectors and their recombinants it was found to be a good host for large pBR322 derivatives. *E. coli* strain JM83 was mainly used for transformation with recombinants of pUC plasmids. The two strains *E. coli* JM101 and W71.18 were both derived from *E. coli* K12 and were used as hosts for recombinants of single stranded phages M13mp8/9 and M13mp18/19, respectively.

2. Culturing conditions

E. coli strains LE392 and JM83 were cultivated either in liquid LB-medium (Miller, 1972) or on LB-plates containing 1.5% (w/v) bacto-agar. For selection of transformed cells ampicillin (100mg/ml in water) was added at a concentration of 50 μ g/ml to liquid medium or of 70 μ g/ml to plates. Amplification of plasmid DNA in *E. coli* was caused by addition of chloramphenicol (34mg/ml in ethanol) at a concentration of 10 μ g/ml to plates.

E. coli strains JM101 and W71.18 were grown in 2 x YT liquid medium or on M9-minimal plates containing glucose (Miller, 1972). Liquid cultures were grown up in Erlenmeyer flasks or Falcon tubes at 37°C with shaking. For long time storage *E. coli* strains were grown to the logarithmic growth phase (absorption at 650 nm 0.3 - 0.5) in the appropriate growth medium and kept frozen at -80°C after addition of 20% (v/v) glycerol. Working strain stocks were stored on agar plates at 4°C for up to one month.

3. Preparation of competent cells

Competent cells were prepared by a simplified procedure involving calcium chloride (Mandel & Higa, 1970). 50 ml cultures of *E. coli* cells were grown up to the logarithmic growth phase (Absorption at 650 nm: 0.3 - 0.5). After incubation on ice for 10 min, the cells were pelleted by centrifugation (Sorvall RT-6000, 3200 rpm, 5 min, 4°C) and carefully resuspended in 25 ml prechilled 0.1 M calcium chloride. The cells were

kept on ice for at least 20 min and again centrifuged as above. Finally, the pellet was taken up in 2.5 ml ice-cold 0.1 M calcium chloride and the competent cells were stored on ice until use (most often for up to 24 h).

4. Transformation of competent cells

Generally, competent *E. coli* cells were transformed with a volume of recombinant plasmid DNA not exceeding 10% of the total volume. After addition of ligation-mix (see 4. 2. 19.) the cells were left on ice for at least 30 min. Cells were then heat-shocked at 42°C for 2 min in order to increase transformation efficiency.

1 ml prewarmed LB-medium was added to transformed *E. coli* LE392 or JM83 followed by incubation at 37°C for 45 min with occasional shaking. Aliquots (up to 0.2 ml) were plated on selective plates and grown overnight at 37°C.

E. coli JM101 and W71.18 transformed with recombinant M13mp18/19 were put back on ice immediately after heat-shock and supplemented prior to plating with 3 ml H-top agar (Miller, 1972) containing 2 mM IPTG, 0.4 mg/ml X-gal, and 200 µl logarithmically growing *E. coli* cells.

5. Identification of recombinant clones

Recombinant clones were identified by colony hybridization (Grunstein & Hogness, 1975). Large numbers of colonies were replicated onto nitrocellulose filters according to Hanahan & Meselson (1980). In order to increase the copy number of recombinant plasmid DNA rapidly growing bacteria were transferred to agar plates containing 10 µg/ml chloramphenicol. *E. coli* cells were lysed and the liberated DNA was bound to the nitrocellulose filters according to Maniatis *et al.* (1982). Filters were prehybridized, hybridized and washed as described in 4. 2. 26.

4. 2. MOLECULAR BIOLOGICAL METHODS

4. 2. 1. General techniques and materials

Glassware and plasticware were sterilized when necessary (e.g. for working with RNA) by rinsing in 0.6 % (v/v) diethylpyrocarbonate (diluted in 70 % (v/v) ethanol) for 3 h and subsequent baking (180°C, 2h) or autoclaving (121°C, 20 min) and drying at 60°C. Only doubly distilled water was used for the preparation of buffers. Solutions were sterilized either by autoclaving (121°C, 20 min) or by filtration through 0.22 µm membrane filters (Millipore GF-C).

Restriction enzymes were purchased from Boehringer, PL, Anglian Biotechnology Ltd. and Amersham. Lysozyme, proteinase K, alkaline phosphatase and bovine serum albumin were obtained from Boehringer. Reverse transcriptase and terminal transferase came from Anglian Biotechnology Ltd.. DNA polymerase I, pancreatic deoxyribonuclease, T4 DNA ligase, T4 polynucleotide kinase, (α -³²P)dCTP and (γ -³²P)ATP were obtained from Amersham. ³⁵S-methionine was from New England Nuclear. Nuclease S1, salmon sperm DNA, wheat germ tRNA and agarose type II came from Sigma. Seaplaque* agarose was purchased from FMC Corp., glass wool from Alltech Inc., nitrocellulose from Schleicher & Schuell. Products for synthesis of oligodeoxynucleotides came from Bachem. Deoxynucleoside triphosphates, Sephacryl S-300 and S-1000, Sephadex G-25 and G-50 were purchased from Pharmacia. Oligo(dT)cellulose was obtained from BRL. All other chemicals were of highest purity available.

4. 2. 2. Isolation of total cellular RNA

Total RNA was extracted from *N. crassa* by the method described by Lucas *et al.* (1977). 1 g lyophilized mycelia were pulverized for 2 sec in a stainless steel waring blender. After addition of 15 ml extraction buffer (100 mM sodium acetate pH 5.2, 1 mM EDTA, 4% (w/v) SDS) the suspension was again blended for 10 sec. The mixture was extracted with

an equal volume phenolmix (49% (v/v) phenol, 49% (v/v) chloroform, 2% (v/v) isoamylalcohol) by blending for 2 min and stirring for 15 min, followed by centrifugation (Sorvall SS34, 10'000 rpm, 10 min, 4°C). The organic phase was reextracted with 7.5 ml extraction buffer (without SDS) by stirring for 15 min and centrifugation as above. Proteinase K was added to the combined aqueous phases at a concentration of 0.1 mg/ml and incubated for 60 min at 25°C. The mixture was then extracted four times with an equal volume of phenolmix. Finally, the RNA pellet was precipitated at -20°C with two volumes of ice-cold ethanol. This procedure could be easily scaled up or down (0.1 - 5 g freeze-dried *N. crassa* mycelia) without any influence on the yield of RNA.

4. 2. 3. Selective precipitation of high molecular weight RNA

Total cellular RNA extracted from 1 g *N. crassa* mycelia was dissolved in 3 ml sterile water. Three volumes of a cold solution containing 4 M sodium acetate pH 6.0, 10 mM EDTA were added and the mixture was allowed to stand in an ice-bath for 10 min. The pellet obtained by subsequent centrifugation (Sorvall SS34, 13'000 rpm, 20 min, 4°C) was again redissolved in water and potassium acetate was added at a final concentration of 0.2 M. Then the high molecular weight RNA was precipitated at -20°C with two volumes of ethanol.

4. 2. 4. Selection of poly(A)RNA by oligo(dT)cellulose chromatography

Oligo(dT)cellulose chromatography was carried out by a modified procedure according to Aviv & Leder (1972). RNA was dissolved in sterile water at a concentration of 1 - 2 mg/ml, heat denaturated at 65°C for 5 min, and rapidly chilled down to 25°C in an ice-bath. After addition of an equal volume of 2 x binding buffer (40 mM Tris HCl pH 7.5, 1 M lithium chloride, 2 mM EDTA, 0.2% (w/v) SDS) the sample was applied to the oligo(dT)cellulose column (bed volume 2 ml) with a flow rate of 1 - 2 ml/min. The non-bound eluate was reapplied once more. The column was

washed thoroughly with binding buffer (20 mM Tris HCl pH 7.5, 0.5 M lithium chloride, 1 mM EDTA, 0.1% (w/v) SDS) until no UV-absorbing material could be detected in the eluate. Bound RNA was then eluted with low salt buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.05% (w/v) SDS) and fractions were collected. By monitoring UV-absorption at 260 nm fractions containing poly(A)RNA were located, pooled, and precipitated at -20°C after addition of one-tenth volume of 4 M sodium chloride and two volumes of cold ethanol.

4. 2. 5. In vitro translation of poly(A)RNA

Cell free synthesis by *in vitro* translation was performed according to Pelham & Jackson (1976). An *in vitro* translation kit (New England Nuclear) containing rabbit reticulocyte lysate as the cell-free system was used. The standard protocol supplied by the manufacturer was followed with 1.5 µg poly(A)RNA isolated from *N. crassa* as template. ³⁵S-methionine (1100 Ci/mmol) was incorporated as a label into *in vitro* translation products.

4. 2. 6. Analysis of RNA on denaturing agarose gels

RNA samples were analyzed on agarose gels containing formaldehyde as described by Maniatis *et al.* (1982). Briefly, 5 - 20 µg RNA were dissolved in 20 µl denaturation buffer (20 mM MOPS pH 7.0, 50 mM sodium acetate, 1 mM EDTA, 12.5 M formamide, 2.2 M formaldehyde), heated at 55°C for 15 min, and quickly chilled on ice. 2 µl 10 x loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromphenol blue, 0.4% (w/v) xylene cyanol) were added and the samples were run on a 1% (w/v) agarose gel in MAE (20 mM MOPS pH 7.0, 50 mM sodium acetate, 1 mM EDTA) containing 2.2 M formaldehyde at constant voltage (3 - 5 V/cm) with buffer recirculation.

4. 2. 7. Northern transfer

RNA was transferred from denaturing agarose gels to nitrocellulose immediately after electrophoresis without prior treatment of the gel (Thomas, 1980). The transfer buffer used was 20 x SSC (0.3 M trisodium citrate pH 7.0, 3 M sodium chloride). After 16 - 24 h transfer was complete and RNA was bound to the filters by baking at 80°C in a vacuum oven for 2 h.

4. 2. 8. Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides were synthesized according to the phosphotriester method (Miyoshi *et al.*, 1980a, b). The synthesis was carried out on a manual solid phase DNA synthesizer (Bachemgentec, Torrance, CA, USA) by exactly following the protocol of the supplier. 15 mg of activated dimer were coupled per step to initially 12 mg resin at 45°C for 25 min. After the last coupling step the oligodeoxynucleotide probes were cleaved from the resin with 0.3 ml 0.5 M tetramethyl guanidinium-2-pyridine aldoximate (prepared by dissolving equimolar amounts of 2-pyridine aldoximate and tetramethyl guanidine in 90% (v/v) pyridine/water) at 45°C for 24 h. The reaction mixture was then filtered through glass wool. After washing the resin with pyridine and ethanol the filtrate was evaporated under vacuum to an oil. This was dissolved in 1 ml 28 - 30 % ammonium hydroxide, sealed in a vial, and incubated at 55°C for 20 h. By this step 3'blocking groups were removed. After evaporation the residue was dissolved in 1 ml 10 mM triethylammonium bicarbonate and applied to a Sephadex G-50 column (1 x 60 cm) equilibrated with the same buffer. The fractions containing DNA (detected by absorption at 260 nm) were lyophilized. In order to remove the 5'blocking groups the oligodeoxynucleotide was treated with 80% (v/v) acetic acid at 25°C for 25 min. After dilution with water the solvent was removed by repetitive lyophilization. The probe was subsequently further purified on a 20% (w/v) polyacrylamide/50% (w/v) urea gel in TBE-S (89 mM Tris base, 89 mM boric acid, 3 mM EDTA). The slowest moving band visualized by UV-shadowing was cut out and extracted with extraction

buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.25 mM sodium chloride) at 50°C for 16 h. Contaminating compounds (e.g. acrylamide residues, urea) were removed by ion exchange chromatography on NACS 52 (BRL) according to the protocol supplied by the manufacturer. After elution with 4 M ammonium acetate the sample was lyophilized and rendered salt free by Sephadex G-25 gel filtration in 10 mM triethylammonium bicarbonate. Oligodeoxynucleotides were stored at -20°C.

4. 2. 9. Primer extension

Oligodeoxynucleotide directed cDNA synthesis was carried out according to the protocols described by Efstratiadis *et al.* (1976) and Nathans & Hogness (1983). Briefly, lyophilized ³²P-labeled oligomer (0.7 pmoles, 3 Ci/mmol) was heated with 1.5 µg poly(A)RNA in 5 µl water at 80°C for 2 min and rapidly chilled on ice for 1 min. 5 µl 10 x FSB (0.5 M Tris HCl pH 8.5, 0.4 M potassium chloride, 80 mM magnesium chloride, 4 mM DTT) were added and the sample was incubated at 25°C for 15 min. Subsequently, 35 µl water, 5 µl 10 mM dNTPs (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP in water, pH 7.0) and 0.5 µl reverse transcriptase were added and the mixture was incubated at 37°C for 90 min. Primer extension was stopped with 2 µl 0.25 M EDTA and 10 µl 1 M sodium hydroxide at 65°C for 30 min. After precipitation with 0.3 M sodium acetate pH 5.2 and 2.2 volumes of ethanol at -20°C, the cDNA synthesized was analyzed on a 6% (w/v) polyacrylamide/42% (w/v) urea gel in TBE-S (89 mM Tris base, 89 mM boric acid, 3 mM EDTA).

The procedure described for primer extension was scaled up five times when the cDNA obtained was subjected to sequence analysis. Furthermore the primer extension products were fractionated by Sephacryl S-300 gel filtration in TES (10 mM Tris HCl pH 7.6, 1 mM EDTA, 0.3 M sodium chloride) and only high molecular weight cDNA was analyzed by the chemical cleavage technique (see 4. 2. 24. 1.).

4. 2. 10. Synthesis of double stranded cDNA

Double stranded cDNA was prepared using Amersham's cDNA synthesis system. The protocol supplied by the manufacturer was strictly followed. Compared to the originally published methods (Okayama & Berg, 1982; Gubler & Hoffman, 1983) Amersham's cDNA synthesis procedure represents a simplified one tube protocol with no intermediate purification steps. First strand synthesis was carried out with 5 µg poly(A)RNA as template in a total volume of 30 µl. After second strand synthesis the cDNA was purified by repetitive phenolic extraction, ether extraction and repetitive precipitation. Double stranded cDNA was cloned without any prior size fractionation as described in 4. 2. 11.

4. 2. 11. Cloning of double stranded cDNA

Double stranded cDNA was cloned into pUC8 plasmid as described by Gubler & Hoffman (1983). Both cDNA and linearized vector (cut with HindII) were tailed with dGTP and dCTP, respectively. After annealing at 58°C for 90 min in annealing buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 150 mM sodium chloride) competent *E. coli* JM83 were transformed as described in 4. 1. 3. 4.

4. 2. 12. Isolation of genomic DNA from *N. crassa*

Fungal genomic DNA was prepared according to the improved method described by Stevens & Metzenberg (1982). Starting with 75 mg lyophilized mycelia usually more than 0.5 mg *Neurospora* DNA with an average length of more than 20 kb (determined by agarose gel electrophoresis) were obtained.

4. 2. 13. Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated by alkaline lysis (Birnboim & Doly, 1979) as follows. 5 ml overnight cultures of *E. coli* cells were pelleted by centrifugation (Sorvall RT-6000, 3200 rpm, 5 min, 4°C), resuspended in 0.2 ml prechilled QSI (25 mM Tris HCl pH 8.0, 50 mM glucose, 10 mM EDTA, 2 mg/ml lysozyme), and left on ice for 30 min. 0.4 ml QSII (0.2 M sodium hydroxide, 1% (w/v) SDS) were added and the mixture was further incubated for 5 min on ice. Chromosomal DNA was precipitated by addition of 0.3 ml QSIII (3 M sodium acetate pH 4.8) and incubation on ice for 60 min. After centrifugation (Sorvall SS34, 10'000 rpm, 15 min, 4°C) plasmid DNA was precipitated from the supernatant with 0.6 volumes isopropanol at -80°C for at least 10 min. The plasmid DNA pellet obtained by centrifugation as above was washed with 70% (v/v) ethanol, air dried for 15 min, redissolved in 50 mM Tris HCl pH 8.0, 0.15 M sodium chloride and reprecipitated with 2.5 volumes of ice-cold ethanol at -20°C. This procedure could easily be scaled up to isolate plasmid DNA from *E. coli* cultures of a volume of 1 l.

4. 2. 14. Purification of covalently closed circular plasmid DNA

1. Sephacryl S-1000 gel filtration

Plasmid DNA isolated by alkaline lysis (4. 2. 13.) was dissolved in TE (10 mM Tris HCl pH 7.6, 1 mM EDTA) and subsequently applied on a Sephacryl S-1000 column equilibrated in the same buffer containing 0.3 M sodium chloride. Maximal amounts applied were 2 µg plasmid DNA per ml bed volume dissolved in a volume not exceeding 5% of the bed volume. The chromatography was carried out with a flow rate of 20 ml/min at room temperature. Collected fractions were analyzed by gel electrophoresis on agarose (see 4. 2. 20.) followed by ethidium bromide staining (Hintermann, *et al.*, 1981). Covalently closed circular DNA containing fractions were pooled and precipitated at -20°C with 2.5 volumes of absolute ethanol.

2. Cesium chloride equilibrium gradient centrifugation

The plasmid DNA pellet obtained from a 500 ml *E. coli* culture was redissolved in 27 ml TE (10 mM Tris HCl pH 8.0, 1 mM EDTA). After addition of 29 g cesium chloride and 2.3 ml ethidium bromide (10 mg/ml in water) the mixture was sealed into 25 x 89 mm polyallomer tubes ('Quick-seal', Beckman) and centrifuged (Beckman VTI 50 rotor, 39 000rpm) for 24 h at 15°C. The covalently closed circular DNA was detected by illumination with a long wave UV-lamp and collected by piercing the tube just below the plasmid band with a syringe (Maniatis *et al.*, 1982). The DNA was freed of ethidium bromide dye by repetitive extraction with sodium chloride saturated isopropanol. After dilution with 4 volumes of water covalently closed circular DNA was precipitated at -20°C with 2.5 volumes of prechilled ethanol.

4. 2. 15. Digestion of plasmid DNA with restriction endonucleases

Generally, restriction endonuclease digestions were carried out using conditions described by Maniatis *et al.* (1982). Enzymes which did not work satisfactory were incubated in the buffers recommended by the manufacturers.

4. 2. 16. Dephosphorylation of DNA fragments

Usually phosphatase treatment of 1 µg DNA was carried out in a volume of 50 µl. The DNA fragment dissolved in 10 µl 10 mM Tris HCl was mixed with 5 µl 10 x CIP buffer (0.5 M Tris HCl pH 9.0, 10 mM magnesium chloride, 1 mM zinc chloride, 10 mM spermidine) and 33 µl water. In order to remove terminal phosphate groups from DNA fragments with 5'protruding ends 1 µl CIP (alkaline phosphatase from calf intestine, 20U/µl) was added and incubated at 37°C for 30 min. Then a second aliquot of CIP was added and incubated at 37°C for a further 30 min. In order to dephosphorylate DNA fragments with blunt ends or recessed 5'termini

repeated incubations for both portions of CIP were 15 min at 37°C and 56°C. CIP was inactivated at 65°C for 30 min in the presence of 5 mM trinitrilo acetic acid. The sample was then extracted twice with equal volumes of buffered phenol (Maniatis *et al.*, 1982), once with water saturated ether and finally precipitated at -20°C with 2 volumes of ethanol.

4. 2. 17. Labeling of 5'ends using T4 polynucleotide kinase

Phosphatase treated DNA fragments (4. 2. 16) were redissolved in 11.5 µl denaturation buffer (10 mM Tris HCl pH 9.5, 0.1 mM EDTA, 1 mM spermidine), heated at 90°C for 2 min, and rapidly chilled on ice. A freeze-dried aliquot of (γ -³²P)ATP (5000 Ci/mmol) was taken up in 1.5 µl 10 x kinase buffer (0.5 M Tris HCl pH 9.5, 100 mM magnesium chloride, 50 mM DTT) and the denaturated DNA was added to this tube. The reaction was started with 2 µl T4 polynucleotide kinase (diluted 1:10 with 50 mM Tris HCl pH 7.6, 10 mM β -mercapto ethanol, 1 mg/ml BSA) and the mixture was incubated at 37°C for 45 min. Excess label was removed by repetitive ethanolic precipitations in the presence of 0.3 M sodium acetate pH 5.2 at -20°C.

Oligonucleotides were labeled without sample denaturation according to the same procedure. Unincorporated (γ -³²P)ATP was separated by Sephadex G-25 gel filtration in 10 mM triethylammonium bicarbonate.

4. 2. 18. Nick-translation

Nick-translation reaction to prepare ³²P-labeled DNA was performed according to Maniatis *et al.* (1982). Usually 1 µg DNA fragment dissolved in 5 µl TE (10 mM Tris HCl pH 7.6, 1 mM EDTA) was mixed with 3 µl unlabeled dNTPs (equal volumes of 1 mM dATP, 1 mM dGTP, 1 mM dTTP), 5 µl 10 x nick-translation buffer (0.5 M Tris HCl pH 7.2, 100 mM magnesium chloride, 1 mM DTT), 2.5 µl BSA (0.1 mg/ml in water), and (α -³²P)dCTP (3000 Ci/mmol). Water was added up to 43 µl. The reaction was started with 5 µl DNase I (0.2 - 0.5 µg/ml in 0.15 M sodium chloride,

50% (v/v) glycerol) and 2 μ l *E. coli* DNA polymerase I and the mixture was incubated at 15°C for 90 min. After addition of 2 μ l 0.5 M EDTA nick-translated DNA was separated from incorporated label by chromatography on Sephadex G-50 (5 ml bed volume) in TE (10 mM Tris HCl pH 8.0, 1 mM EDTA).

4. 2. 19. Ligation of DNA fragments with compatible ends

Typical ligation reactions contained equimolar amounts of DNA fragments to be joined together or about five fold excess of DNA fragments to be inserted into linearized vectors. Usually the reactions were carried out in a total volume of 10 μ l containing 1 μ l 10 x ligase reaction buffer (0.1 M Tris HCl pH 7.5, 0.1 M magnesium chloride, 0.5 M sodium chloride), 1 μ l 10 mM ATP, and 1 μ l 50 mM DTT. For sticky ends 0.1 units T4 DNA ligase were added and the mixture was incubated at 14°C for 4 - 12 h. Blunt ends were ligated with 0.5 units T4 DNA ligase at 4°C for 24 h. The ligation-mix was either used directly for transformation (see 4. 1. 3. 4.) or stored on ice after heat inactivation of the ligase (65°C, 10 min).

4. 2. 20. Analysis and recovery of DNA fragments by agarose gel electrophoresis

DNA fragments with a length of > 200 bp were analyzed i.e. size fractionated, using horizontal slab gels (Fangmann, 1978). Electrophoresis was carried out at constant voltage (10 - 15 V/cm) on gels of concentrations ranging from 0.7 - 2%. The buffers used were TAE (40 mM Tris base, 40 mM sodium acetate, 20 mM EDTA) or TBE-K (50 mM Tris base, 50 mM boric acid, 22.5 mM EDTA). Before lading 1 μ l 6 x loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added to 5 μ l sample. After electrophoresis gels were stained with gentle agitation for 10 - 15 min in ethidium bromide (0.5 μ g/ml in water). DNA bands were visualized by transillumination with a UV-source.

For analytical purposes agarose type II was used and stained DNA bands were detected by short wavelength UV-irradiation (Vilber-Lourmat TF-20-C transilluminator). If desired gels were photographed with a Polaroid film type 667 through a Kodak Wratten 22A filter.

For preparative purposes DNA fragments were run on 'low gelling temperature' agarose (Seaplaque*) and visualized by irradiation with long wavelength UV-source (Vilber-Lourmat TF-35-L transilluminator). DNA bands were cut out and gel slices were melted at 65°C for 20 min after addition of 10 volumes extraction buffer (20 mM Tris HCl pH 7.5, 1 mM EDTA, 0.2 M sodium chloride). Subsequently, DNA fragments were purified by Elutip-d chromatography (Schleicher & Schuell) according to the protocol supplied by the manufacturer.

Alternatively, high molecular weight DNA fragments (> 2 kb) were recovered by phenol extraction. Agarose was melted after addition of an equal volume of 0.8 M sodium chloride at 65°C for 15 min, cooled down to 37°C, and extracted twice with buffered phenol (Maniatis *et al.*, 1982) and twice with water saturated ether. Finally the DNA was precipitated at -20°C with 2 volumes of ethanol.

4. 2. 21. Analysis and recovery of DNA fragments by non denaturing polyacrylamide gel electrophoresis

Low molecular weight DNA fragments (< 500 bp) were analyzed on 5 - 20% (w/v) polyacrylamide slab gels (Maniatis *et al.*, 1982). Electrophoresis was performed at constant voltage (20 V/cm) in TBE-K (50 mM Tris base, 50 mM boric acid, 22.5 mM EDTA). Prior to loading samples were diluted 1:6 with 6 x loading dye (see 4. 2. 20.). Non labeled DNA bands were visualized by ethidium bromide staining (as described in 4. 2. 20.). ³²P-labeled DNA fragments were detected by autoradiography (Kodak XAR-5 films).

For recovery the DNA band of interest was cut out and the gel was crushed by pressing through a 2 ml plastic syringe. 1 ml ELU-X (0.5 M

ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (w/v) SDS) was added and the suspension incubated at 37°C for 16 h with shaking (115 rpm). Gel fragments were separated by filtration through glass wool and washed with 1 ml ELU-X. The DNA was precipitated at -80°C after addition of EDTA at a concentration of 10 mM, sodium acetate pH 5.2 at 0.3 M and 2 volumes of ethanol. To remove remaining traces of acrylamide the pellet was resuspended in 0.3 M sodium acetate, 0.1 mM EDTA, centrifuged (Sorvall SS34, 10'000 rpm, 15 min, 4°C), and the DNA reprecipitated from the supernatant.

4. 2. 22. Strand separation of DNA fragments

Strand separation of ^{32}P -labeled DNA fragments (< 2 kb) was achieved by electrophoresis of denatured DNA through 5-10% (w/v) polyacrylamide gels (Maxam & Gilbert, 1977). The fragments were dissolved in TE (10 mM Tris HCl pH 7.6, 1 mM EDTA) and diluted 1:5 with 5 x SSB (50% (v/v) glycerol, 1.5 M sodium hydroxide, 5 mM EDTA, 5% (w/v) bromophenol blue, 5% (w/v) xylene cyanol). The samples were heated at 90°C for 2 min and immediately chilled on an ice bath. Electrophoresis was carried out in TBE-K (50 mM Tris base, 50 mM boric acid, 22.5 mM EDTA) at constant voltage (0.5 V/cm) for 16 h at 4°C. The separated DNA strands detected by autoradiography were cut out and recovered as described in 4. 2. 21.

4. 2. 23. Analysis of DNA fragments by denaturing polyacrylamide gel electrophoresis

Products obtained by nucleotide sequencing of DNA fragments (see 4. 2. 24) were analyzed on polyacrylamide gels containing 50% (w/v) urea. Buffers used were TBE-M (134 mM Tris base, 45 mM boric acid, 2.5 mM EDTA) for 5% gels, TBE-S (89 mM Tris base, 89 mM boric acid, 3 mM EDTA) for 6% and 7% gels, and TBE-K (50 mM Tris base, 50 mM boric acid, 22.5 mM EDT) for 20% gels. The gels were preelectrophoresed in the appropriate running buffer at constant voltage (15 V/cm) for 12 h. Prior

to loading of samples the gel was preheated to 45°C with constant current (25 mA for 5%, 6%, 7% and 15 mA for 20% gels) for 1 h. Samples were denaturated in formamide dye (0.1% (w/v) xylene cyanol, 1 mM EDTA in deionized formamide) for at least 2 min at 90°C and immediately applied. Electrophoresis was carried out at constant voltage as described for preelectrophoresis. Finally, 5%, 6% and 7% gels were soaked in fixing solution (10% (v/v) methanol, 10% (v/v) acetic acid) for 10 min, vacuum dried (Bio-Rad model 1125B slab gel drier), and exposed for autoradiography. 20% gels were exposed directly after electrophoresis.

4. 2. 24. Nucleotide sequencing

1. Chemical cleavage technique

DNA fragments labeled at a single 5'end were obtained by strand separation (4. 2. 22.) or by cutting with a restriction endonuclease after labeling with T4 polynucleotide kinase (4. 2. 17.). They were subjected to chemical sequencing according to the protocol described by Maxam & Gilbert (1980) and subsequently analyzed on denaturing 7% and 20% gels (4. 2. 23.).

2. Chain-termination technique

Single-stranded templates for chain-termination sequencing (Sanger, *et al.*, 1979) were obtained by molecular cloning of isolated subfragments in the single stranded bacteriophage vectors M13mp18 and M13mp19 (Norrander *et al.*, 1983). Kits from Amersham were used for M13 cloning and sequencing and the protocols supplied by the manufacturer were strictly followed. Single stranded DNA templates were prepared as described by Messing (1983), annealed with the 17mer 'universal primer' (Duckworth *et al.*, 1981) and subjected to sequencing using (α -³²P)dCTP as label. Sequencing products were analyzed on denaturing 5% and 6% polyacrylamide gels (4. 2. 23.).

4. 2. 25. Southern transfer

Southern transfer (Southern, 1975) was performed with minor modifications as described by Maniatis *et al.* (1982). Briefly, by soaking agarose gels in each solution for 30 min DNA was depurinated by 0.25 M HCl, denaturated by 0.5 M sodium hydroxide, 1.5 M sodium chloride and neutralized by 0.5 M Tris HCl pH 7.5, 1.5 M sodium chloride. The DNA was transferred to nitrocellulose during 2 - 16 h using 20 x SSPE (0.2 M sodium hydrogen phosphate-monohydrate pH 7.4, 22.5 mM EDTA, 3 M sodium chloride) as buffer system. After transfer the nitrocellulose was incubated in 6 x SSC (0.3 M sodium citrate pH 7.0, 3 M sodium chloride) for 5 min and baked in a vacuum oven at 80°C for 2 h.

4. 2. 26. Hybridization of immobilized nucleic acids

Nitrocellulose filters with bound DNA or RNA were sealed in plastic bags containing prehybridization (0.2 ml/cm²) or hybridization solution (0.05 ml/cm²) and incubated with shaking at the appropriate temperature.

1. Hybridization with labeled oligonucleotides

Dissociation temperature T_d of a oligodeoxynucleotide was estimated by the semiempirical 'Wallace-rule': $T_d = (2 \times \text{number of A-T bp}) + (4 \times \text{number of G-C bp})^\circ\text{C}$ (Wallace *et al.*, 1979). Prehybridizations and hybridizations were performed at a temperature of $>10^\circ\text{C}$ below T_d for 2 h and 16 h, respectively. Prehybridization solution was 6 x NET (0.9 M sodium chloride, 6 mM EDTA, 0.18 M Tris HCl pH 7.5), 0.5% (v/v) Nonidet P40 and 100 µg/ml denatured salmon sperm DNA. Hybridization solution contained 6 x NET, 0.5% (v/v) Nonidet P40, 250 µg/ml wheat germ tRNA and the ³²P-labeled oligodeoxynucleotide (10⁶ cpm/ml). After hybridization the filters were washed several times in a large volume of 6 x SSC (3 M sodium chloride, 0.3 M sodium citrate pH 7.0) at a temperature below T_d and exposed for autoradiography.

2. Hybridization with nick-translated DNA

Hybridizations of nitrocellulose with bound DNA or RNA to DNA fragments labeled by nick-translation were performed using the conditions described by Maniatis *et al.* (1982). 1 ml of ^{32}P -labeled DNA fragment in 10 mM Tris HCl pH 8.0 was denaturated prior to hybridization by mixing with 0.2 ml 1 M sodium hydroxide and incubation for 10 min at room temperature. The solution was neutralized with 0.2 ml 1 M Tris HCl pH 8.0 and 0.2 ml 1 M HCl.

4. 2. 27. Nuclease S1 mapping

Nuclease S1 mapping was carried out according to the protocol described by Weaver & Weissmann (1979). Briefly, DNA was coprecipitated with *N. crassa* poly(A)RNA and yeast carrier RNA. The pellet was resuspended in 35 μl hybridization solution (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M sodium chloride, 80% (v/v) deionized formamide), sealed in a capillary tube, and denaturated in a water bath at 90°C for 10 min. Formation of DNA-RNA hybrids was achieved by cooling the water bath slowly down to the appropriate hybridization temperature (depending on the G+C content of the RNA and estimated according to Maniatis *et al.*, 1982) and incubation for 12 - 16 h. Then the mixture was transferred to 100 μl of S1 buffer (30 mM sodium acetate pH 4.5, 1 mM zinc sulfate, 0.25 M sodium chloride). 20 units nuclease S1 were added and digestion was carried out for 45 min at 25°C. Finally, nucleic acids were extracted once with 50% (v/v) phenol/50% (v/v) chloroform and once with water saturated ether.

4. 3. PROTEIN CHEMICAL METHODS

4. 3. 1. Materials

Sephadex G-25, G-50, G-100 and DEAE-Sephadex were purchased from Pharmacia. Trypsin (treated with TPCK) and chymotrypsin were obtained from Worthington. Cyanogen bromide was from Pierce. Iodo(2- ^{14}C)acetic acid was purchased from Amersham. Hydroxyapatite was produced as described by Tiselius *et al.* (1956). Glutaraldehyde-fixed *Staphylococcus aureus* cells were prepared according to Kessler (1975). ^{14}C -labeled M_r marker proteins were obtained from New England Nuclear.

4. 3. 2. Isolation of *N. crassa* laccase

Extracellular *N. crassa* laccase was isolated essentially as described by Lerch *et al.* (1978) from the culture medium collected 96 h after induction. 10 l filtrated medium were evaporated under vacuum to 0.5 l and subsequently dialyzed against 4.5 l of 50 mM sodium phosphate pH 6.0 at 4°C for 16 h with one buffer change after 4 h. The brownish dialyzate was concentrated once more by evaporation to a volume of < 50 ml, centrifuged (Sorvall SS34, 20'000 rpm, 30 min, 4°C) and applied on a Sephadex G-100 column (5 x 150 cm) equilibrated in 50 mM sodium phosphate pH 6.0. The slightly yellow fractions containing laccase activity were pooled and applied on a hydroxyapatite column (2.5 x 6 cm) equilibrated in the same buffer. Laccase was eluted with a linear gradient formed by 200 ml of each 50 mM and 0.5 M sodium phosphate pH 6.0. Again pools of laccase containing fractions were composed, dialyzed against 4 l of 50 mM sodium phosphate pH 6.0 as above and applied on a DEAE-Sephadex column (0.9 x 5 cm) connected with another hydroxyapatite column (0.9 x 5 cm). After elution from DEAE-Sephadex with 50 mM sodium phosphate pH 6.0 a blue band was observed stacking on the hydroxyapatite column. Laccase was then eluted in a single step with 0.5 M sodium phosphate pH 6.0 and dialyzed once more against 0.1 M sodium

phosphate pH 6.0. The purified enzyme was stored at -20°C without loss of activity for several months.

Apolaccase was prepared by extended dialysis (at least 24 h) of the native enzyme against 5% (v/v) formic acid at 4°C and subsequently lyophilized.

4. 3. 3. Laccase assay

Laccase activity was determined spectrophotometrically using L-3,4-dihydroxyphenylalanine (5 mM in 0.1 M sodium phosphate pH 6.0) as substrate (Lerch *et al.*, 1978). One unit was defined as an increase of absorption of 0.2/min at 436 nm and 30°C.

4. 3. 4. S-carboxymethylation

Chemical modification of laccase by S-carboxymethylation was performed according to Gurd (1967). In order to reduce disulfide bonds lyophilized apolaccase was dissolved in 1 M Tris HCl pH 8.0, 6 M guanidine HCl at a concentration of 0.2 µmol/ml, purged with argon for 10 min and incubated with DTT (tenfold molar excess relative to sulfhydryl groups) for 1 h at 25°C. Subsequently iodoacetic acid (tenfold molar excess relative to DTT) was added and the solution stirred for 1 h at room temperature in the dark. If labeling of cysteine residues was desired the mixture was first reacted with iodo(2-¹⁴C)acetic acid (40-60 mCi/mmol) for 1 h, followed by incubation with non labeled iodoacetic acid for the same time. The modification reaction was stopped with a six-fold molar excess of β-mercaptoethanol (relative to iodoacetic acid) for 15 min. After addition of 0.25 volumes of concentrated formic acid S-carboxymethylated apolaccase was desalted by gel filtration on Sephadex G-25 (1.5 x 75 cm) in 7% (v/v) formic acid. Protein containing fractions were stored at 4°C.

4. 3. 5. Cyanogen bromide cleavage

Lyophilized, S-carboxymethylated apolaccase (0.15 $\mu\text{mol/ml}$) was dissolved in 70% (v/v) formic acid and reacted with 200fold excess of cyanogen bromide over methionine for 24 h at 25°C in the dark. The mixture was then diluted tenfold with ice-cold water, lyophilized and size fractionated by passage through a Sephadex G-50 column (2.5 x 150 cm) equilibrated in 7% (v/v) formic acid.

4. 3. 6. Enzymic digestions

Tryptic and chymotryptic digestions were performed in 0.1 M ammonium bicarbonate pH 8.5 at 37°C with 2% (w/w) trypsin (TPCK-treated) or chymotrypsin, respectively. After 1 h a second aliquot of enzyme was added and the mixture further incubated for 3 h at 37°C.

4. 3. 7. Isolation of peptides

Peptides were purified by reversed phase HPLC using either a Vydac C4 column (0.46 x 25 cm, 5 μm particle size, 30 nm pore size, The Separations group, Hesperia, CA, USA) or an Aquapore RP-300 column (C-4, 0.46 x 25 cm, 7 μm particle size, 30 nm pore size, Brownlee Laboratories, Santa Clara, CA, USA). The solvent system used was buffer A 0.1% (v/v) TFA and buffer B, same as A containing 60% (v/v) acetonitrile.

4. 3. 8. Amino acid analysis

Samples were hydrolyzed in 6 M HCl *in vacuo* at 110°C for 22 h and subsequently analyzed either on a Durrum D-500 amino acid analyzer (Durrum Instrument Corp.) or by dansylation (5-dimethylaminonaphtalene-1-sulfonylation) followed by HPLC (de Jong *et al.*, 1982).

4. 3. 9. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Acrylamide concentrations used were 3% (w/v) in the stacking gel and 10% (w/v) in the separation gel. Electrophoresis was performed at constant current (stacking gel: 15 mA; separation gel: 25 mA), and afterwards, the gels were stained in Coomassie blue solution (0.25% (w/v) Coomassie blue, 9.2% (v/v) acetic acid, 45.6% (v/v) methanol) for 30 min and subsequently soaked in destaining solution (10% (v/v) acetic acid, 10% (v/v) propanol) with gentle agitation. Finally, gels were dried on a vacuum drier (Bio-Rad model 1125B slab gel drier) at 60°C.

In order to detect radioactively labeled proteins gels were incubated for 3 h in enhancer solution (Enlightning, New England Nuclear), for 1 h in water and for 15 min in 10% (v/v) glycerol immediately after electrophoresis. They were dried as described above and exposed to X-ray films (Kodak XAR-5) at -80°C.

4. 3. 10. Western transfer

Proteins were transferred electrophoretically from SDS polyacrylamide gels to nitrocellulose membranes using Bio-Rad Trans Blot^R Cell. Blotting was performed according to the protocol supplied by the manufacturer at constant voltage (60 V) for 5 h at 4°C. The buffer used contained 25 mM Tris HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol.

Proteins bound to nitrocellulose were detected with amido black (Towbin *et al.*, 1979; Gershani & Palade, 1983).

Laccase was assayed by a double antibody EIA system (Bio Rad) using horse radish peroxidase and 4-chloro-1-naphtol according to the instruction manual of the supplier. Rabbit anti-laccase antiserum against denatured laccase was diluted 1:1000 and incubated for 12 - 16 h with gentle agitation. Best sensitivity was obtained when protein A-horse radish peroxidase conjugate was diluted 1:10'000.

4. 3. 11. Immunological detection of *in vitro* synthesized laccase

In vitro synthesized ^{35}S -labeled laccase was detected by immunological precipitation under denaturing conditions with rabbit anti-laccase antiserum and *Staphylococcus aureus* bearing protein A, followed by SDS polyacrylamide gel electrophoresis and autofluorography. Briefly, *in vitro* translation products in a volume of 25 μl were denaturated with 60 μl 10% (w/v) SDS for 5 min at 95°C. The solution was then diluted with 6 ml Triton buffer (50 mM Tris HCl pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 1% (w/v) Triton X-100) and preincubated for 1 h with 100 μl of a suspension of 10% (w/v) *S. aureus*. The supernatant obtained by centrifugation (Sorvall RT-6000, 2600 rpm, 15 min, 20°C) was incubated with 100 μl of rabbit antiserum against denatured laccase for 20 h at room temperature. Immunoprecipitation was achieved by addition of 200 μl *S. aureus* suspension (see above), continuing incubation for 1 h, and centrifugation as described. The resulting pellet was washed four times with Triton buffer containing 0.1% (w/v) SDS and 1 mg/ml methionine. The *S. aureus* antibody-antigen complexes were dissociated by resuspending the pellet in 10 μl water and 20 μl loading buffer, followed by incubation at 95°C for 4 min. Finally, the supernatant obtained by centrifugation was analyzed by SDS polyacrylamide gel electrophoresis and autofluorography (see 4. 3. 9.)

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6. ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
DEAE	diethylaminoethyl
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
cdNA	complementary DNA
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immuno assay
EPR	electron paramagnetic resonance
h	hour
HPLC	high pressure ('performance') liquid chromatography
IPTG	isopropyl- β -D-thiogalactopyranoside
kb	kilo base pairs
kD	kilo daltons
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
M _r	molecular weight
pI	isoelectric point
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
SDS	sodium dodecylsulfate
sec	second
SSC	3 M sodium chloride, 0.3 M sodium citrate pH 7.0
TFA	trifluoroacetic acid
TPCK	L-1-tosylamido-2-phenylethylchloromethyl-ketone
Tris	tris(hydroxymethyl)aminomethane
U	international unit
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- β -galactoside

For the abbreviation of genetic markers see Miller (1972).
For the one-letter abbreviation of amino acids see IUPAC-IUB (1968).

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